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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	S. Ananth Karumanchi et al.	Confirmation No.:	6646
Serial No.:	10/624,809	Art Unit:	1647
Filed:	July 21, 2003	Examiner:	Ian D. Dang
Customer No.:	21559		
Title:	METHODS OF DIAGNOSING AND TREATING PRE-ECLAMPSIA OR ECLAMPSIA		

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DECLARATION OF DR. S. ANANTH KARUMANCHI

1. I am an inventor of the subject matter that is described and claimed in the above-captioned patent application.
2. I am a nephrologist in the Center for Vascular Biology at Beth Israel Deaconess Medical Center and an Assistant Professor of Medicine, Obstetrics and Gynecology at Harvard Medical School. I am a co-founder of Nephromics, Inc., which holds a license to

the above-referenced application.

3. I have read the specification of the above-referenced patent application and the Office Action dated June 30, 2006.

4. The above-referenced application features methods for the diagnosis of pre-eclampsia or eclampsia that include the detection of sFlt-1, free PlGF, or free VEGF polypeptides. The diagnostic methods can be performed using biological samples that include bodily fluids, cells, or tissue samples from the subject.

5. In response to the Examiner's assertion that the use of amniotic fluid is not enabled by the specification, I submit that amniotic fluid is readily obtained by the clinician using standard procedures and that PlGF, VEGF, and sFlt-1 can be detected in amniotic fluid samples. Amniotic fluid is obtained through amniocentesis, a procedure which is standard in the art and is typically performed between 15 and 18 weeks of pregnancy. sFlt-1, free PlGF, and free VEGF can be detected using the same techniques described in the specification and known in the art for detection of these polypeptides in serum, urine, blood, or other bodily fluid samples.

6. With regard to the Examiner's assertion that PlGF may not be able to cross the membrane barriers isolating amniotic fluid from the body, I submit that sFlt-1, VEGF, and PlGF have been previously detected in amniotic fluid. Banks et al., *Molecular Human Reproduction* 4:377-386 (1998) and Hornig et al., *J. Immunol. Methods* 226:169-177 (1999), attached herein as Exhibits A and B, respectively, describe the detection of sFlt-1 in amniotic fluid sample from women with normal pregnancies undergoing amniocentesis. Vuorela-Vepsalainen et al., *Hum. Reprod.* 14:1346-1351 (1999), attached herein as Exhibit C, describes the detection of VEGF and PlGF in amniotic fluid using an ELISA. These three articles demonstrate the detection of sFlt-1, PlGF, and VEGF in amniotic fluid prior to the filing of the above-referenced application and provide evidence that the polypeptides can cross the barriers isolating the amniotic fluid from the body.

7. In response to the Examiner's assertion that the use of endothelial cells, leukocytes, monocytes, and cells derived from the placenta are not enabled by the specification, I submit that these cells are readily obtained using techniques known in the art prior to the filing of the above-referenced application. Further, the methods described in the specification for the detection of sFlt-1, free VEGF, and free PlGF can be readily adapted for the detection of these polypeptides in a sample of endothelial cells, leukocytes, monocytes, or cells derived from the placenta. For example, monocytes can be obtained from a sample of peripheral blood mononuclear cells and cells derived from

the placenta can be obtained through chorionic villus sampling (CVS), a procedure in which a small sample of cells from the placenta are obtained and used to screen for chromosomal abnormalities and genetic disorders. The presence of sFlt-1, VEGF, and PlGF has been demonstrated in a variety of cell types including, for example, placental tissue and cells, endothelial cells, and monocytes. For example, Barleon et al., *Angiogenesis* 4:143-154 (2001), attached herein as Exhibit D, demonstrate the presence of sFlt-1 in human peripheral blood monocytes and in endothelial cells (ECs) from a variety of tissues including human dermal microvascular ECs, human umbilical vein ECs, human kidney venous ECs, human kidney microvascular ECs, human renal microvascular ECs, and human umbilical artery ECs. In addition, as indicated by the nomenclature, VEGF, or vascular endothelial growth factor, is highly expressed in the endothelium, and PlGF, or placental growth factor, was initially isolated from placental tissue.

8. In the experiment described below, which was carried out in my laboratory and under my supervision, I show that sFlt-1 polypeptide can be detected in monocytes using the techniques described in the specification. For this experiment, peripheral blood mononuclear cells (PBMCs), rich in monocytes, were isolated from normal and pre-eclamptic patients and used to measure the levels of sFlt-1 and fragments thereof. Protein extracts were prepared from the PBMCs and both Flt-1 and sFlt-1 levels were analyzed by western blots using an antibody that recognizes the N-terminus of Flt-1 protein (a region

common to both proteins). The results of this experiment show increased Flt-1 and sFlt-1 levels in the monocytes from pre-eclamptic patients as compared to the level found in normal patients (Exhibit E). In addition several bands that had a faster migration than full-length sFlt-1 were detected. These faster migrating bands may be degradation products, alternatively spliced isoforms, enzymatic cleavage products, or other forms of sFlt-1. The levels of sFlt-1 detected in PBMCs of a subject sample are compared to the levels of sFlt-1 detected in PBMCs of a reference sample, as claimed, for example, in claims 45 or 50. A skilled artisan would be able to apply these methods to any cell type or tissue type in which sFlt-1, free VEGF, or free PlGF is expressed.

9. A feature of the invention is that the levels of free PlGF polypeptides are decreased during pre-eclampsia or eclampsia. Experiments described below and carried out in my laboratory and under my supervision have clearly demonstrated that our detection methods specifically measure the free form of PlGF. Thus, the decrease in PlGF we have observed in connection with pre-eclampsia is a decrease in free PlGF.

10. To demonstrate the specificity of the ELISA kits for the detection of free PlGF in serum from pre-eclamptic women, we performed a standard curve using recombinant PlGF protein in the absence or in the presence of two different doses of recombinant human sFlt-1 (sFlt1-Fc) using the ELISA kit used for the experiments described in the

specification. The results are shown in Figure 2b of Maynard et al. (*J. Clin. Invest.* 111:649-658 (2003), attached herein as Exhibit F), a paper from my laboratory on which all three inventors of the above-referenced application are authors. The graph shown in Figure 2b demonstrates that the level of PlGF detected by the ELISA significantly decreases in the presence of increasing levels of recombinant sFlt-1. The interference of sFlt-1 with PlGF measurement confirms that the PlGF detected is free PlGF and the decrease in the levels of PlGF detected in the presence of increasing levels of recombinant sFlt-1 is due to the fact that as sFlt-1 is added, more of the PlGF binds to sFlt-1 and is therefore not detected by the ELISA that is specific for only the free form of PlGF. These experimental results demonstrate that the detection methods used in the experiments described in the specification of the above-referenced application specifically detect the free form of PlGF.

11. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

11/29/06

Date



S. Ananth Karumanchi, M.D.

## Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1

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Angiogenesis is essential in physiological processes including ovulation, implantation and pregnancy. One of the most potent regulators is the cytokine vascular endothelial growth factor (VEGF). We provide evidence for a novel pregnancy-associated soluble variant of the VEGF receptor Flt-1. VEGF ranged from undetectable to 157.3 pg/ml (mean 49.9 pg/ml, SD 48.4 pg/ml) in plasma samples from normal volunteers ( $n = 10$ ), but was undetectable in plasma from pregnant women ( $n = 12$ ) and amniotic fluid ( $n = 10$ ). Recoveries of spiked VEGF were poor in pregnancy-related samples, indicating the presence of VEGF-binding activity which was confirmed using biosensor and chromatographic techniques. Partial purification and protein sequencing indicated a novel soluble form of Flt-1 with a subunit size of 150 kDa. Normally present as a multimeric structure of ~400–550 kDa, complexes of 600–700 kDa were formed following binding of multiple VEGF molecules. Reverse transcriptase polymerase chain reaction of Flt-1 in placenta, amnion, chorion, human umbilical vein endothelial cells and cord blood samples produced bands of the predicted sizes but failed to identify any additional RNA species, and possible reasons for this are discussed. Soluble Flt-1 may be important in regulating the actions of VEGF in angiogenesis and trophoblast invasion and may have therapeutic implications in diseases with inappropriate angiogenesis such as proliferative retinopathies and cancer.

**Key words:** angiogenesis/Flt-1/pregnancy/soluble receptor/VEGF

### Introduction

Angiogenesis, the development of new blood vessels from an existing vascular bed, is essential in many physiological processes including ovulation, placentation and embryogenesis. Several factors have been identified which have angiogenic activity but one of the most potent and specific, which was originally identified as a secreted product of tumour cells and has both angiogenic and vasculogenic activity, is vascular endothelial growth factor (VEGF) also known as vascular permeability factor (VPF) and vasculotropin (reviewed by Ferrara, 1996; Thomas, 1996; Ferrara and Davis-Smyth, 1997). The pivotal role of VEGF in angiogenesis is clearly illustrated by gene knockout models with abnormal blood vessel development and embryonic lethality resulting from the loss of even a single VEGF allele (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996).

Structurally, VEGF is a member of the platelet-derived growth factor family with other related members including VEGF-related factor (VRF) or VEGF-B, VEGF-C, VEGF-D (Grimmond *et al.*, 1996; Joukov *et al.*, 1996; Olofsson *et al.*, 1996; Paavonen *et al.*, 1996; Yamada *et al.*, 1997) and placenta growth factor (Maglione *et al.*, 1991; Hauser and Weich, 1993; Maglione *et al.*, 1993). The active form of VEGF is a homodimer of 34–46 kDa, with alternative exon splicing

producing four different isoforms of 121, 165, 189 and 206 amino acids (monomeric size), the latter three of which have heparin-binding activity (Houck *et al.*, 1991; Tischer *et al.*, 1991). Many cell types produce VEGF with VEGF<sub>165</sub> being the predominant soluble isoform and the two larger forms being almost exclusively cell- or extracellular matrix-associated (Houck *et al.*, 1992).

Two high-affinity tyrosine kinase receptors for VEGF have been identified and cloned, namely KDR (kinase insert domain-containing receptor, or the murine homologue Flk-1) and the *fms*-like tyrosine kinase Flt-1 (Shibuya *et al.*, 1990; Matthews *et al.*, 1991; Terman *et al.*, 1991; de Vries *et al.*, 1992; Millauer *et al.*, 1993), which are found predominantly but not exclusively on endothelial cells. Both receptors have an extracellular region containing seven immunoglobulin (Ig)-like domains, a single transmembrane domain and a cytoplasmic consensus tyrosine kinase sequence. Although Flt-1 appears to mediate a greatly reduced response in terms of tyrosine phosphorylation and effects on endothelial cell growth compared with KDR, the importance of both receptors is indicated by the lethality *in utero* of either gene knockout model (reviewed by Ferrara 1996; Thomas 1996; Ferrara and Davis-Smyth, 1997).

VEGF has been clearly implicated in the angiogenesis and vasculogenesis occurring during pregnancy, with protein or

mRNA being variably demonstrated in villous trophoblast, endothelial cells, stromal cells, fetal macrophages within the villi (Hofbauer cells), invading extravillous trophoblast, maternal macrophages and glandular epithelium within the decidua (Sharkey *et al.*, 1993; Jackson *et al.*, 1994; Clark *et al.*, 1996; Shiraishi *et al.*, 1996). In a comprehensive study involving analysis of both protein and mRNA for VEGF and its receptors throughout pregnancy, VEGF was found in the villous trophoblast, endothelial cells, stromal cells and macrophages, tending to co-localize with its receptor Flt-1 (Clark *et al.*, 1996). Although found in extravillous trophoblast throughout pregnancy, expression of Flt-1 was temporally and spatially regulated, with patchy expression in villous trophoblast being down-regulated in mid-gestation and increasing at term. In contrast, the VEGF receptor KDR was localized exclusively to endothelial cells, particularly in the villous stroma adjacent to areas of trophoblast proliferation during the first trimester around the time of vascularization of the villi (Clark *et al.*, 1996; Vuckovic *et al.*, 1996).

VEGF mRNA has been shown to be present in many epithelial, but not endothelial, tissues during fetal development in animals and humans (Breier *et al.*, 1992; Jakeman *et al.*, 1993; Shiffen *et al.*, 1994). Flk-1/KDR is present in endothelial cell precursors at the earliest embryonic developmental stages (Millauer *et al.*, 1993; Quinn *et al.*, 1993) with Flt-1 mRNA being selectively expressed in the endothelial cells of fetal and adult mice (Peters *et al.*, 1993).

In view of the angiogenic actions of VEGF and its effects in increasing microvascular permeability (Senger *et al.*, 1983; Connolly *et al.*, 1989; Roberts and Palade, 1997), the regulation of VEGF activity is thus likely to be critical in ensuring a successful pregnancy. Although the mechanisms involved in regulating cellular expression of VEGF are gradually being elucidated, and implicate a variety of factors including cytokines, steroid hormones, mutated tumour suppressor genes and hypoxia (Smith, 1996; Ferrara and Davis-Smyth, 1997; Greb *et al.*, 1997), little is known about factors governing the bioavailability of released VEGF which is equally crucial in regulating systemic concentrations. Binding of VEGF to  $\alpha_2$ -macroglobulin *in vitro* has been reported (Soker *et al.*, 1993) and a soluble 110 kDa form of the Flt-1 receptor has been identified in the supernatant of cultured human umbilical vein endothelial cells (Kendall and Thomas, 1993; Kendall *et al.*, 1996). This soluble Flt-1 variant is able to inhibit VEGF-induced endothelial cell proliferation *in vitro* and hence may be a naturally occurring antagonist of VEGF. The mRNA for this soluble form has been found in placental tissue, appearing to predominate over the full-length form, at least at term (Barleon *et al.*, 1994), although the soluble protein has yet to be demonstrated in biological fluids *in vivo*. RNA corresponding to two additional novel soluble variant forms of Flt-1 has also been identified in ovarian carcinoma cells, human umbilical vein endothelial cells and human chorion although the properties of these proteins if expressed *in vivo* are not yet known (Boockock *et al.*, 1995).

We describe here the identification *in vivo* of a 150 kDa pregnancy-associated VEGF-binding protein which is a novel soluble variant of Flt-1 and may function in the normal

regulation of VEGF activity during pregnancy. This finding may further our understanding of the normal regulation of angiogenesis during pregnancy but may also have therapeutic implications for conditions such as cancer where the inhibition of angiogenesis may be desirable.

## Materials and methods

All chemicals used were molecular biology grade or Analar grade as appropriate and were purchased from Sigma (Poole, UK) or BDH (Poole, UK) unless otherwise indicated.

### Patient samples

Venous blood samples (clotted) were collected from 10 healthy volunteers (seven female, three male) and 12 pregnant women (8–32/40 weeks post-last menstrual period) attending routine antenatal clinics. After centrifugation at 1500 g for 10 min, the plasma was removed, and stored at  $-80^{\circ}\text{C}$ . Amniotic fluid samples from pregnant women undergoing amniocentesis who subsequently had normal pregnancies were centrifuged at 450 g for 5 min, the supernatant removed and stored at  $-80^{\circ}\text{C}$  until used for assay or purification studies. Pieces of tissue were obtained from a first-trimester placenta following termination of pregnancy and from three term placentae together with two amnion and chorion samples at the time of elective Caesarean section and were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction of RNA. At the time of one elective Caesarean, a sample of cord blood was also obtained, the mononuclear cells isolated by density-gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway) as per the manufacturer's instructions, and stored at  $-80^{\circ}\text{C}$  until extraction of RNA. Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared from umbilical cords obtained from women undergoing normal delivery. Briefly, the umbilical vein was cannulated, flushed through with Hanks' balanced salt solution (HBSS) (Life Technologies, Paisley, UK) and filled with HBSS containing dispase II (5 mg/ml; Boehringer Mannheim, Lewes, UK). Following incubation at  $37^{\circ}\text{C}$  for 15 min, cells were harvested by centrifugation and grown in Clonetics microvascular endothelial cell growth medium (EGM-MV) (TCS Biologicals, Botolph Claydon, UK) at  $37^{\circ}\text{C}$  in a 95% air/5%  $\text{CO}_2$  incubator for subsequent extraction of RNA as described below.

### Immunoassay of VEGF

VEGF concentrations of the plasma samples were determined using a sandwich ELISA with a detection limit of 9 pg/ml (R & D Systems, Oxford, UK). Samples were also spiked with 150 pg/ml of recombinant human VEGF<sub>165</sub> (Peprotech, London, UK) and assayed for VEGF to determine recovery. Amniotic fluid samples from 10 pregnant women (14–22/40 weeks post-LMP) who had a subsequent normal pregnancy were also assayed for VEGF and reassayed following spiking with 220 ng/ml of VEGF<sub>165</sub> to determine recovery. The differing amounts of VEGF used for spiking plasma and amniotic fluid samples were determined on the basis of being able to saturate the binding capacity in at least some of the samples and thus allow a relative comparison of binding activity between different types of samples.

### Biosensor analysis of VEGF-binding activity

VEGF-binding activity of the amniotic fluid samples was also assessed using a Pharmacia Biacore X biosensor (Pharmacia, Milton Keynes, UK). Briefly, VEGF<sub>165</sub> [1921 resonance units (RU) =  $\sim 2.4$  ng] was immobilized on a CM5 (carboxymethyl) sensor chip using amine coupling chemistry by injection of VEGF (1  $\mu\text{l}$  of 9  $\mu\text{g}$  VEGF/ml) in 10 mM acetate coupling buffer pH 4.8 at a flow rate of 1  $\mu\text{l}/\text{min}$ .



min, following activation of the chip surface with 0.05 M *N*-hydroxysuccinimide/0.2 M *N*-ethyl-*N'*-(dimethyl-aminopropyl)-carbodiimide according to the manufacturer's protocols. Binding was assessed by injection of 25 µl of each amniotic fluid sample (5 µl/min) using an HBS flow buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% v/v surfactant P20) at a temperature of 20°C. The chip was regenerated using 10 µl of 10 mM glycine, pH 2.0 between samples. Specificity of binding was checked by prior absorption of the amniotic fluid samples (100 µl) with either 100 ng of VEGF or interleukin (IL)-6 (irrelevant control). Two amniotic fluid samples (200 µl) were also subjected to fractionation by size separation using a Superose 6 H/R 10/30 column on a Pharmacia fast protein liquid chromatography (FPLC) system using an elution buffer of 0.05 M sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and flow rate of 0.4 ml/min with 150 µl fractions being assayed for binding activity using the biosensor and by measuring recovery of spiked VEGF (+150 pg/ml) using the ELISA.

#### Radiolabelled VEGF-binding studies

To measure the extent of VEGF binding, 200 µl of a pooled amniotic fluid sample, plasma samples from a normal volunteer and a pregnant woman were each incubated with 24.5 fmol (980 pg) of [<sup>125</sup>I]VEGF (specific activity = 2030 Ci/mmol; Amersham, Chalfont, UK) for 30 min at room temperature before undergoing fractionation by size separation as described above. Similar samples but containing an excess (0.5 µg) of unlabelled VEGF<sub>165</sub> were also treated in the same way and all fractions (500 µl) were then assayed for radioactivity.

Cross-linking studies were performed using bis-sulphosuccinimidyl suberate (BSS; Pierce, Chester, UK). Briefly, amniotic fluid samples (100 µl) were incubated with [<sup>125</sup>I]VEGF as above, BSS was then added to a final concentration of 1 mM and incubated for 2 min at 4°C before the reaction was stopped by adding glycine solution to a final concentration of 0.1 M. Samples (10 µl) were then subjected to electrophoresis [6% for native polyacrylamide gel electrophoresis (PAGE), 4–15% for sodium dodecyl sulphate (SDS)–PAGE, reducing and non-reducing]. Gels were fixed briefly in 30% v/v methanol, 10% v/v acetic acid for 10 min, drained, wrapped in cling-film and exposed to pre-flashed Kodak X-OMAT/AR film at –70°C for 2–7 days.

#### Purification of the VEGF-binding protein

To obtain preliminary information about the molecular structure of the binding protein, a pool of amniotic fluid prepared from ~100 samples (total volume of ~450 ml) was concentrated by ultrafiltration using a Gyrosep<sup>TM</sup> 300 stirred cell with a membrane of 100 000 mol. wt cut-off (Intersep Filtration Systems, Wokingham, UK). An ammonium sulphate fraction (Englard and Seifter, 1990), between 30 and 55% saturation (judged from Biosensor readings to contain at least two-thirds of the VEGF binding activity) was then prepared and dialysed against phosphate-buffered saline (PBS) to yield a final volume of 6 ml. Following dilution with PBS (1:1), 400 µl was then incubated with 3.5 µg of VEGF coupled to cyanogen bromide-activated sepharose (40 µg VEGF per ml of swollen gel prepared according to the manufacturer's instructions). Following incubation for 1 h at room temperature, the supernatant was assayed by biosensor to ascertain that all the VEGF-binding activity was bound to the Sepharose beads. The Sepharose was then washed twice in PBS, and boiled for 4 min in 100 µl of SDS–PAGE sample buffer (Laemmli, 1970). A similar sample was prepared in a reducing SDS–PAGE sample buffer containing 2-mercaptoethanol. Controls included the use of blank Sepharose to evaluate non-specific carry-over, and the prior absorption of the amniotic fluid with 5 µg of VEGF or an irrelevant cytokine (IL-6) before addition to the VEGF–Sepharose.

Samples (20 µl) were then electrophoresed using a 4–20% gradient gel (Biorad, Hemel Hempstead, UK), silver-stained using a Plus One Protein staining kit (Pharmacia) and dried.

For further purification of the binding protein, amniotic fluid batches (~200–400 ml) were concentrated as described above and a 35–70% ammonium sulphate preparation post-dialysis (20–30 ml) was injected onto a 1 ml HiTrap heparin–Sepharose column (Pharmacia) on a Pharmacia FPLC system at a flow rate of 1 ml/min. After washing with PBS, the bound material was eluted from the column using PBS with a gradient of 0.15 to 2 M NaCl. Fractions (1 ml) were collected, salt exchanged into PBS using Centricon 30 concentrators (Amicon, Watford, UK), the VEGF-binding activity was determined using the Biosensor and the purity of the fractions checked by gradient gel electrophoresis as before. For determination of the protein sequence, fractions containing the highest amount of binding activity were concentrated and electrophoresed under reducing conditions. Proteins were transferred to Immobilon P membrane (Millipore, Watford, UK) by electroblotting at 15 V for 45 min using a Tris–glycine transfer buffer (0.048 M Tris, 0.039 M glycine, 5% v/v methanol and 0.01% w/v SDS, pH 9.2). For determination of the extent of glycosylation, similar fractions which contained the greatest amount of VEGF-binding activity were chemically deglycosylated with the agent trifluoromethanesulphonic acid using a Glycofree<sup>TM</sup> deglycosylation kit (Oxford Glycosystems, Oxford, UK) and examined by gradient gel electrophoresis.

#### Protein sequencing

The electroblotted proteins were stained with Ponceau S (0.05% w/v in 30% v/v aqueous methanol/0.1% v/v acetic acid) using the rapid-staining protocol (Coull and Pappin, 1990). The dried, stained protein (apparent mol. wt 150 kDa) was then digested *in situ* with trypsin (Boehringer, modified) and the peptides extracted with 1:1 v/v formic acid:ethanol (Sutton *et al.*, 1995). One 0.2 µl aliquot (~5% of the total digest) was sampled and directly analysed by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry using a LaserMat 2000 mass spectrometer (Thermo Bioanalysis, Hemel Hempstead, UK; Mock *et al.*, 1992). A second 0.2 µl aliquot was quantitatively esterified using 1% v/v thionyl chloride in methanol and also analysed by MALDI to provide acidic residue composition (Pappin *et al.*, 1996). Native and esterified peptide masses were then screened against the MOWSE peptide mass fingerprint database (Pappin *et al.*, 1993). The remaining digested peptides (90% of total digest) were then reacted with *N*-succinimidyl-2-(3-pyridyl)acetate (SPA) in order to enhance b-ion abundance and facilitate sequence analysis by tandem mass spectrometry (Sherman *et al.*, 1995). Dried peptide fractions were treated with 7 µl 1% w/v *N*-succinimidyl-2-(3-pyridyl)acetate in 0.5 M HEPES (pH 7.8 with NaOH) containing 15% v/v acetonitrile for 20 min on ice. The reaction was terminated by 1 µl heptafluorobutyric acid (HFBA) and the solution immediately injected onto a capillary reverse-phase column (300 µm×15 cm) packed with POROS R2/H material (Perseptive Biosystems, MA, USA) equilibrated with 2% v/v acetonitrile/0.05% v/v trifluoroacetic acid (TFA) running at 3 µl/min. The adsorbed peptides were washed isocratically with 10% v/v acetonitrile/0.05% v/v TFA for 30 min at 3 µl/min to elute the excess reagent and HEPES buffer. The derivatized peptides were then eluted with a single step gradient to 75% v/v acetonitrile/0.1% v/v formic acid and collected in a single 4 µl fraction. The derivatized peptides were then sequenced by low-energy collision-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitted with a nanoelectrospray source (Hunt *et al.*, 1986; Wilm and Mann, 1996). CAD was performed using 2.5 mTorr argon with collisional offset voltages between –18 and –28 V. The production spectra were collected with Q3 scanned at 500 amu/s.

### RNA extraction

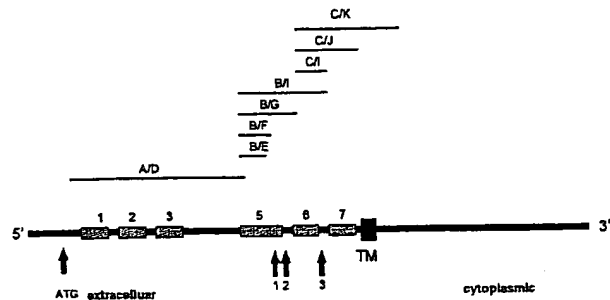
Snap-frozen tissue was ground in a pestle and mortar on dry ice and RNA extracted (Chomczynski and Sacchi 1987) using Ultraspec (Biogenesis, Poole, UK) as per the manufacturer's instructions. The resultant air-dried RNA pellets were resuspended in water and stored at  $-80^{\circ}\text{C}$ . Concentration and  $A_{260}/A_{280}$  ratio was checked and integrity assessed by examination of the ribosomal bands after gel electrophoresis. All materials used for RNA work were autoclaved before use or certified RNAase-free by the manufacturer.

### Northern blotting

7.5–10  $\mu\text{g}$  of RNA in sample buffer was denatured by heating at  $65^{\circ}\text{C}$  for 5 min. Samples were electrophoresed at 100 V in a 1% agarose gel containing 6.5% w/v formaldehyde in MOPS buffer, together with size markers (0.24–9.5 kb RNA ladder; Gibco BRL, Glasgow, UK). The RNA was visualized using a UV transilluminator and then blotted onto Hybond-N nylon membrane (Amersham) by capillary elution in  $20\times$  saline-sodium citrate (SSC) overnight. The RNA was then cross-linked to the membrane by exposure to UV light for 10 min. Probes for Flt-1 were prepared from the PCR products of the reactions of primer pairs B/H, B/E, C/J and C/I described below, and used as a cocktail. Probes were labelled with 50  $\mu\text{Ci}$  of [ $^{32}\text{P}$ ]dCTP using a Rediprime kit (Amersham) with unincorporated radioactivity being removed using Sephadex G-50 (Nick<sup>TM</sup> column, Pharmacia). The blot was prehybridized for 2–4 h at  $42^{\circ}\text{C}$  in prehybridization buffer [50% v/v deionized formamide,  $5\times$  SSC,  $5\times$  Denhardt's solution, 0.1% (w/v) SDS, 250  $\mu\text{g}/\text{ml}$  salmon sperm DNA] before addition of the probes and hybridization overnight at  $42^{\circ}\text{C}$ . After washing twice for 30 min in  $2\times$  SSC containing 0.2% w/v SDS at  $60^{\circ}\text{C}$  and a further  $2\times$  in  $0.2\times$  SSC as above, the blot was exposed to Kodak Biomax film at  $-80^{\circ}\text{C}$ . The blot was stripped by soaking in boiled SDS (0.1% w/v) and reprobed for  $\beta$ -actin.

### Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was prepared from RNA samples using the Reverse Transcription system (Promega, Southampton, UK). Briefly, 1  $\mu\text{g}$  of RNA (10  $\mu\text{l}$  volume) was denatured at  $65^{\circ}\text{C}$  for 5 min. RT components were added to yield final reaction conditions of 5 mM  $\text{MgCl}_2$ , 10 mM Tris-HCl (pH 9.0 at  $25^{\circ}\text{C}$ ), 50 mM KCl, 0.1% Triton X-100, 1 mM each dNTP, 20 U recombinant RNasin, 15 U avian myeloblastosis virus (AMV) reverse transcriptase and 0.5  $\mu\text{g}$  Oligo (dT)<sub>15</sub> primer in a total volume of 20  $\mu\text{l}$  per tube. Samples were then heated at  $42^{\circ}\text{C}$  for 15 min,  $99^{\circ}\text{C}$  for 5 min and  $4^{\circ}\text{C}$  for 5 min. The cDNA samples were then either stored at  $-20^{\circ}\text{C}$  or used for PCR as follows. Primers were designed to cover the whole of the extracellular region of Flt-1, overlapping and focusing especially on the region where the splicing sites for the three previously described soluble variants lie (Figure 1; Kendall and Thomas, 1993; Boocock *et al.*, 1995). Hot-start PCR was carried out in a Biometra thermocycler using 2  $\mu\text{l}$  of cDNA, 10 nmol of each dNTP, ThermoPol buffer with a final  $\text{Mg}^{2+}$  concentration of 2 mM, and 1 U of Deep Vent polymerase (all New England Biolabs, Hitchin, UK), in a total volume of 50  $\mu\text{l}$ . PCR was carried out for 40 cycles (1 min at  $95^{\circ}\text{C}$ , 1 min at  $55^{\circ}\text{C}$ , 90 s at  $72^{\circ}\text{C}$ ) with a final extension of 10 min at  $72^{\circ}\text{C}$ . Parallel reactions were also carried out with RNA preparations which had been treated in the same way but in the absence of RT enzyme (RT negative). PCR was also carried out for glyceraldehyde-3-phosphate dehydrogenase as a positive control under the same conditions using primers 5'-TCGGAGTCAACGGATTGG-3' and 5'-GCATTGCTGATGATCTTGAG-3'. Reaction products were then electrophoresed in 1.5% (w/v) agarose gels and visualized with ethidium bromide.



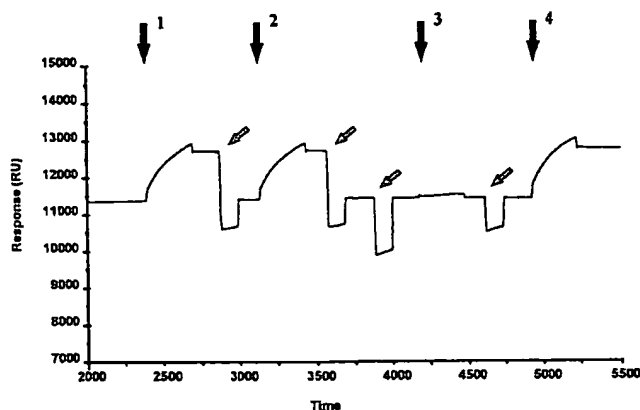
**Figure 1.** Schematic diagram showing the localization of the regions amplified by the primer pairs used for the reverse transcription-polymerase chain reaction analysis of Flt-1. The sequences of the forward (A–C) and reverse primers (D–J) were as follows, with the numbering from the start of the published cDNA sequence (Genbank Accession No. X51602, Shibuya *et al.*, 1990) where the start ATG codon is at base 250, with the exception of primer H which is located on a sequence specific to one of the soluble variants (Kendall and Thomas, 1993): (A) 5'-CTGCTCAGCTGTCTGCTTCT-3' (289–308), (B) 5'-ACCCACTGGGCAGCAGACAAA-3' (1580–1600), (C) 5'-GCACTACAGTATTAGCAAGCAA-3' (2049–2070), (D) 5'-ATACCATATGCGGTACAAGT-3' (1606–1625), (E) 5'-GCCATGCGCTGAGTGATGC-3' (1760–1778), (F) 5'-ATTCTAGAGTCAGCCACAACCA-3' (1814–1835), (G) 5'-CCGCAGTAAATCCAAAGTAAC-3' (2008–2028), (H) 5'-CTTTGTGTGGTACAATCATTCC-3', (I) 5'-ACTGAGGTTTCGCGAGGAGGTAT-3' (2232–2253), (J) 5'-TTGTCCGAGGTTCTTGAACAGT-3' (2488–2510), and (K) 5'-AGTCCGCGACGTAGGTGATT-3' (2801–2820). The theoretical locations of the 7 Ig-like domains, extracellular, transmembrane (TM) and cytoplasmic regions are indicated. The points at which the alternative sequence starts for the three previously published soluble forms of Flt-1 (1,2: Boocock *et al.*, 1995; 3: Kendall and Thomas, 1993) are indicated by arrows. The region amplified by primer pair B/H is not indicated, as primer H binds a sequence unique to one of the known variants (Kendall and Thomas, 1993).

### cDNA sequencing of RT-PCR products

For each primer pair reaction, PCR products generated as above from between two and six tissue samples were purified from the agarose gels using a Qiaquick gel extraction kit (Qiagen, Crawley, UK). Each product was then sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA polymerase, FS (Perkin Elmer, Warrington, UK) and a Perkin Elmer ABI377 automated sequencer according to the manufacturer's instructions.

### Results

The VEGF concentrations of plasma from normal volunteers ranged from undetectable ( $<9$  pg/ml) to 157.3 pg/ml with a mean value of 49.9 pg/ml (SD 48.4 pg/ml). The mean recovery of added VEGF was 86.3% (SD 13%). In contrast, VEGF was undetectable in all plasma samples from pregnant women and recoveries of spiked VEGF ranged from 0% ( $n = 9$ ) to a maximum of 25% ( $n = 3$ ). VEGF was also undetectable in all amniotic fluid samples examined with the mean recovery of the high VEGF spike (220 ng/ml) being 44.0% (SD 24.5%). The absence of detectable VEGF and the poor recoveries in the pregnancy-related samples indicated the presence of VEGF-binding molecules.

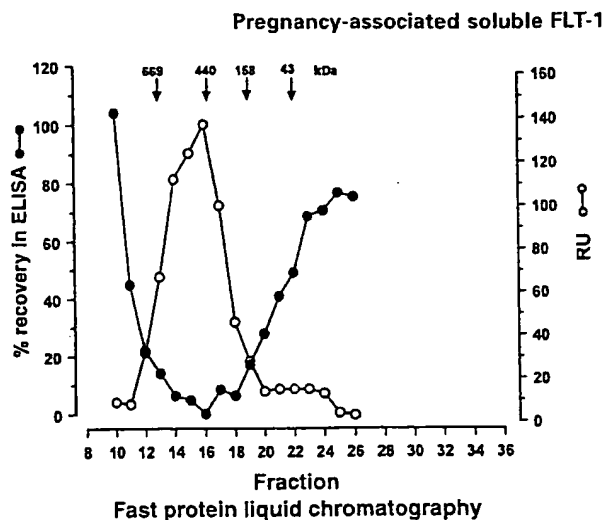


**Figure 2.** Vascular endothelial growth factor (VEGF)-binding activity in amniotic fluid detected using a VEGF<sub>165</sub>-coated CM5 chip with a Pharmacia Bialite biosensor as described in the text. Response is defined as resonance units (RU). Arrows 1–4 indicate injection of an amniotic fluid sample with injections 3 and 4 following prior absorption of the sample with VEGF or IL-6 (100 ng/100  $\mu$ l) respectively. Regeneration of the VEGF chip is indicated by shaded arrows.

The presence of VEGF-binding activity in amniotic fluid was confirmed using the biosensor (range 171–1257 RU) with a significant correlation being found between these values and the VEGF-binding activity of the amniotic fluid samples as assessed indirectly using recovery studies with the ELISA ( $r = -0.852$ ,  $P = 0.002$ ). Prior incubation of a sample with excess VEGF but not an irrelevant cytokine abolished this binding activity (Figure 2). When the fractionated amniotic fluid sample was assayed for binding activity using both methods, good agreement was found with the main broad band of activity eluting with a peak molecular size of ~400–550 kDa (Figure 3).

Size fractionation of normal plasma incubated with radiolabelled VEGF showed a major peak of unbound VEGF eluting at 40–45 kDa (Figure 4a). Traces of radioactivity were also present in fractions 12–18 but this was deemed to be non-specific as it was present when human serum albumin was used in place of the sample and presumably represents aggregates of VEGF. When the pregnancy-associated plasma samples or the amniotic fluid were examined (Figure 4b,c), a similar peak was seen but in addition a large band of activity peaking at ~600–700 kDa was seen which was displaceable by the addition of excess unlabelled VEGF but not by the addition of an irrelevant cytokine (IL-6).

When the VEGF–Sepharose bound proteins were electrophoresed, specific VEGF-binding bands were apparent. Under non-reducing conditions (Figure 5a), elution of the VEGF-sepharose (lane 2) yielded a band at 150 kDa and a diffuse 200–250 kDa band when compared with proteins non-specifically bound to sepharose alone (lane 1). Prior absorption of the amniotic fluid sample with VEGF, but not with IL-6, abolished these bands (lanes 3 and 4). Under reducing conditions (Figure 5b), the corresponding analysis of the proteins bound showed a strong band at 150 kDa with a fainter band at 110–120 kDa. This 150 kDa band was also present in the high mol. wt



**Figure 3.** Size separation of an amniotic fluid sample using a Superose 6 Fast protein liquid chromatography (FPLC) column with detection of vascular endothelial growth factor binding activity by recovery in enzyme-linked immunosorbent assay (closed circles) or by biosensor (RU: resonance units, open circles). The elution profile of molecular size markers is indicated by arrows.

fractions (400–550 kDa) containing VEGF-binding activity following size separation.

Analysis of the cross-linked radiolabelled VEGF-bound proteins in amniotic fluid under native conditions showed a single broad smear of activity which was displaceable with excess unlabelled VEGF. Under non-reducing conditions, two specific bands of ~185–205 and 280 kDa were seen which were displaceable with unlabelled VEGF, whereas, under reducing conditions, only the lower of these two specific bands was seen (results not shown).

Purification of the binding protein by VEGF affinity chromatography was not pursued as we were unable to elute the protein successfully. Similar problems were encountered using lectin columns, with particularly strong binding seen with wheat germ agglutinin indicating the presence of *N*-acetyl glucosamine residues. Using heparin–Sepharose, the binding protein bound completely and was eluted as one of the main proteins at a NaCl concentration of 0.8–1.2M (Figure 6). Following deglycosylation, the 150 kDa VEGF-binding protein was reduced in size to ~115 kDa (Figure 6). The main additional contaminating band (~50 kDa) at this stage was later identified as antithrombin-III. Subsequent attempts to improve the purification of the binding protein have resulted in loss of activity. From biosensor measurements and an estimate of total protein from absorbance values at 280 nm, the maximum recovery of binding activity following ultrafiltration, ammonium sulphate fractionation and heparin–Sepharose purification is 10%, with a 300–500-fold depletion in total protein. From the signal strength obtained during protein sequencing, the amount of the protein in a single blotted band derived from ~100 ml of initial amniotic fluid is 250–500 ng which, with a maximum recovery of 10%, indicates a minimum initial concentration of 25–50 ng/ml.

Peptide mass fingerprint (PMF) analysis of native and esterified tryptic peptides from the blot provisionally identified

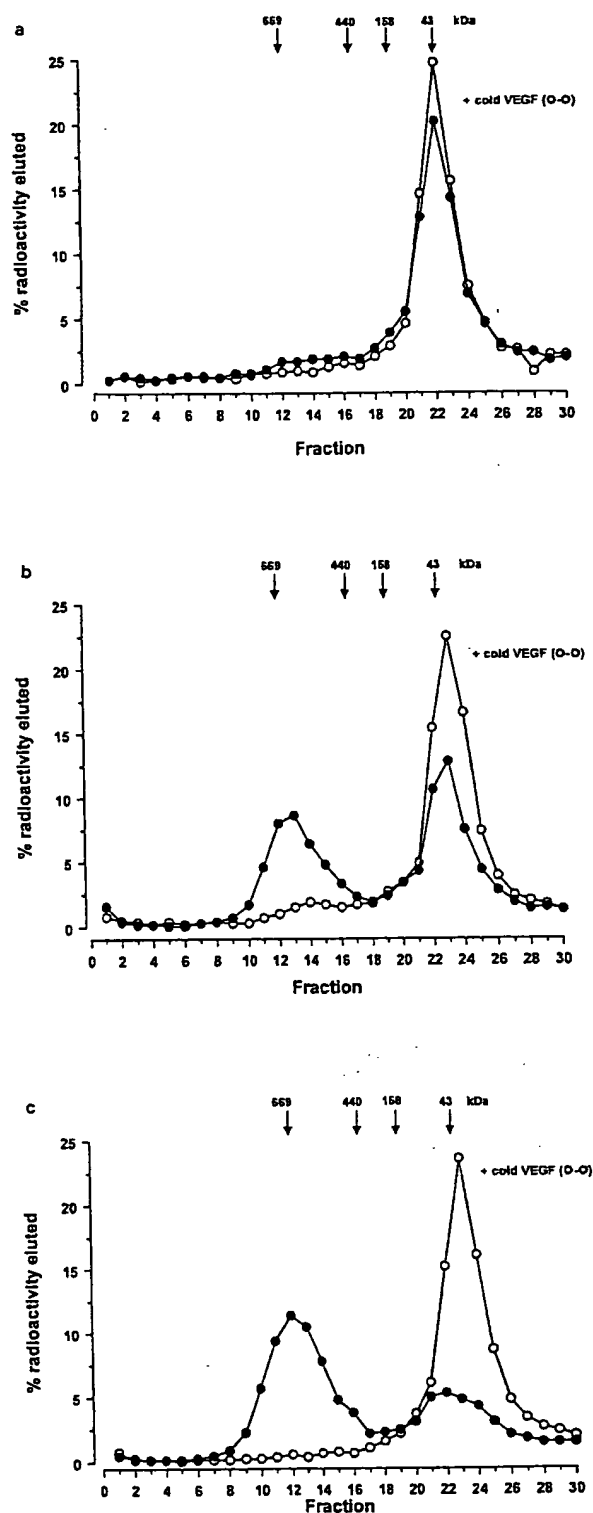


Figure 4. Elution profile of [ $^{125}$ I]vascular endothelial growth factor (VEGF) (closed circles) from a Superose 6 FPLC column following incubation with (a) a plasma sample from a healthy volunteer, (b) a plasma sample from a pregnant woman, and (c) an amniotic fluid sample. The elution profile following incubation with excess unlabelled VEGF (0.5  $\mu$ g) is indicated by the open circles. The elution profile of molecular size markers is indicated by arrows.

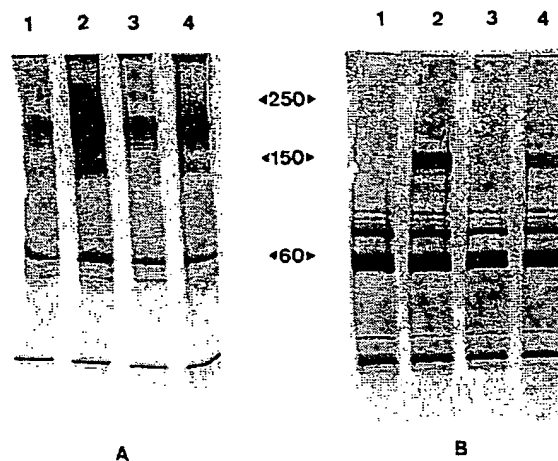


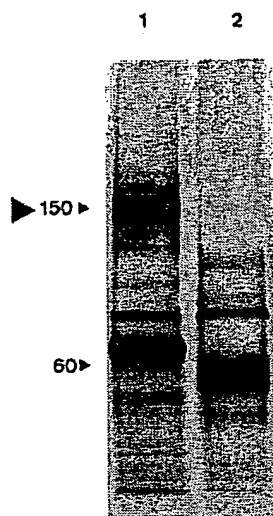
Figure 5. Electrophoresis [under (A) non-reducing and (B) reducing conditions] of amniotic fluid proteins following release from Sepharose (lane 1) or vascular endothelial growth factor (VEGF)-conjugated Sepharose (lanes 2–4). Samples in lanes 3 and 4 had undergone prior absorption with VEGF and interleukin-6 (5  $\mu$ g/400  $\mu$ l of ammonium sulphate-precipitated proteins prepared as described in Materials and methods) respectively. The positions of size markers are indicated (values in kDa).

the 150 kDa protein as vascular endothelial growth factor receptor (VEGFR, Flt-1). Five complete peptide sequences were then obtained by nanoelectrospray tandem mass spectrometry following derivatization with SPA to improve b-ion abundance as described.

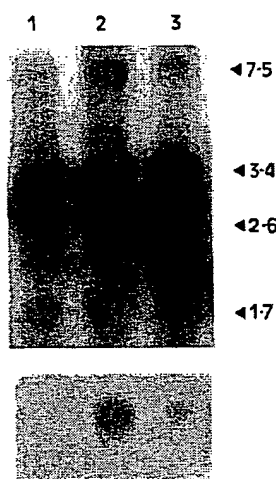
AVSSFPDPAXYPXGSR	434–449	(partial 5th Ig-like domain)
NVVTGEEXXQK	640–650	(partial 6th Ig-like domain)
DVTWXXR	586–593	(6th Ig-like domain)
XDQSNSHANXFYSVXTXDK	282–300	(3rd Ig-like domain)
AFPSPEVVWXX	361–371	(between 3rd and 5th Ig-like domains)

where X is Leu or Ile (L or I), which are isomers and cannot be distinguished by low-energy collisional fragmentation. All five peptide sequences correspond exactly to sequences for tryptic peptides from human VEGFR (Flt-1). All sequenced peptides were in the range from residues 282–650 of the reported sequence (Shibuya *et al.*, 1990). Despite extensive searching, no peptide sequences could be obtained that corresponded to peptides from the C-terminal half of the reported Flt-1 sequence.

Northern blotting showed three main bands of ~7.5, 3.4 and 2.6 kb with the latter two predominating (Figure 7) and a weaker band at 1.4 kb. Strong signals were seen in both the term and first trimester placental samples examined, with relatively weak bands in the amnion and chorion samples. No bands were detected for the cord blood or HUVEC samples although a strong signal was obtained for actin. RT-PCR yielded positive results with bands of the size predicted for all samples examined for each primer pair (Figure 8). Sequencing confirmed the bands to be identical to the published sequence (Shibuya *et al.*, 1990) in each case examined. Occasional additional bands were sequenced and shown to be unrelated and the result of mis-priming.



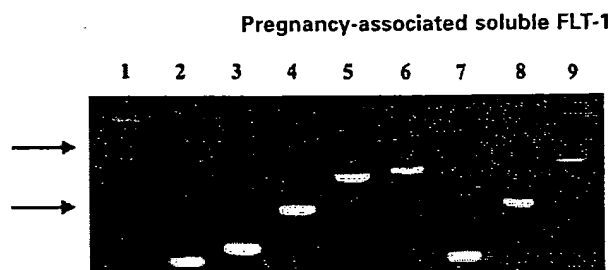
**Figure 6.** Silver-stained SDS-PAGE under reducing conditions of amniotic fluid proteins following ultrafiltration, ammonium sulphate precipitation and heparin-Sepharose chromatography as described in the Materials and methods section. Lane 1 = before deglycosylation, lane 2 = post-deglycosylation. The positions of size markers are indicated (kDa). The large arrowhead indicates the protein band used for protein sequencing.



**Figure 7.** Northern blot of Flt-1 transcripts in human chorion, first trimester placenta and term placenta (lanes 1-3 respectively). The sizes of the transcripts are indicated by the values on the right (kb). The lower panel indicates the signal from the actin probe for each sample.

## Discussion

This is the first study to demonstrate the existence *in vivo* of a soluble VEGF-binding protein, subsequently identified as a novel variant form of the VEGF receptor Flt-1. This variant appears to be pregnancy-associated, being present in plasma from pregnant women and in much higher concentrations in amniotic fluid, whilst undetectable, at least in terms of VEGF-binding activity, in normal plasma samples. Its biological function is not yet clear, but, as already illustrated, VEGF has been implicated as a key angiogenic factor during pregnancy both in placental and fetal development, and it is tempting to



**Figure 8.** Representative reverse transcriptase-polymerase chain reaction results from a term placenta sample for each of the Flt-1 primer pairs as described in the Materials and methods section. Lanes 1-9 show the results with primer pairs A/D, B/E, B/F, B/G, B/H, B/I, C/I, C/J and C/K respectively. Arrows indicate the position of 0.5 and 1.0 kb.

speculate that this soluble receptor plays a role in the regulation of this activity. The regulation of VEGF may also be crucial not only in terms of its angiogenic actions but also with regard to other roles. Elevated systemic concentrations of VEGF have been reported in patients with pre-eclampsia (Sharkey *et al.*, 1996), which may account for the increase in microvascular permeability and hence the ensuing clinical sequelae. A possible role for VEGF in the regulation of trophoblast activity has been proposed with the demonstration that VEGF stimulated DNA synthesis and tyrosine phosphorylation of mitogen-activated protein in the trophoblastic cell line BeWo (Charnock-Jones *et al.*, 1994). This is also supported by the observation that in human trophoblasts, VEGF/Flt-1 mediate calcium-dependent nitric oxide release, which has been suggested to play a role in regulating trophoblast invasion which is defective in pre-eclampsia (Ahmed *et al.*, 1997). Additionally trophoblastic VEGF mRNA has been found to be reduced in women with pre-eclampsia (Cooper *et al.*, 1996).

The size of full-length glycosylated Flt-1 is ~180 kDa, indicating a contribution from glycosylation of ~30 kDa, although glycosylation of Flt-1 is not necessary for VEGF binding (Barleon *et al.*, 1997). Cross-linking studies of cell extracts showed VEGF-Flt-1 complexes of 220-225 kDa (Vaisman *et al.*, 1990; Soker *et al.*, 1996) with larger complexes of ~400 kDa (Gitay-Goren *et al.*, 1996) interpreted as dimerized receptors. In one study, reduction caused an apparent increase in intensity of lower mol. wt complexes of ~170 and 195 kDa (Vaisman *et al.*, 1990), indicating that they may be derived from the larger complex by reduction of disulphide bonds. Whether this is due to release of complexed VEGF or another protein is not clear. The soluble Flt-1 molecule described in this study has an apparent mol. wt of 150 kDa on a reducing gel, ~35 kDa of which is due to glycosylation, and appears to be present in biological fluids as a multimeric complex of 400-550 kDa. The radiolabelled VEGF binding and size separation studies showed the apparent size of the VEGF-complexed binding protein to be 600-700 kDa, compared with a size of 400-550 kDa for the free multimeric binding protein. This supports the binding of several VEGF molecules per multimeric complex. The demonstration of VEGF-binding activity indicates that it is likely that this novel Flt-1 variant contains the 2nd Ig-like domain, as this is essential for VEGF and placenta growth factor (PlGF) binding (Davis-Smyth *et al.*, 1996; Barleon *et al.*, 1997). The possibility exists that this

complex may be stabilized at least in part by interactions with VEGF as the 4th Ig-like domain of full-length Flt-1, which from protein sequencing data is known to be present in the soluble variant described here and is thought to contain the region involved in supporting or stabilising VEGF-induced dimerization of Flt-1 (Barleon *et al.*, 1997). This is thought to occur following VEGF binding through contact sites located at both ends of the VEGF homodimer (Keyt *et al.*, 1996).

Three soluble forms of Flt-1 have previously been described which lack the transmembrane and cytoplasmic regions (as illustrated in Figure 1). The first of these was originally cloned from a HUVEC cDNA library (Kendall and Thomas, 1993) and has a cDNA coding sequence identical to that of the full-length molecule up to bp 2218, after which the truncated form encodes a unique 31 amino acid C-terminus generated by read-through into an intron until encountering a premature translation stop codon (a 2.3 kb coding sequence in total). This variant contains the first six of the seven extracellular Ig-like domains and has a theoretical mass of ~75 kDa. However, it is expressed in baculovirus as an 85–90 kDa protein and is found in the supernatant of HUVEC as a 110 kDa protein, presumably due to glycosylation (Kendall *et al.*, 1996). The molecule inhibits the mitogenic activity of VEGF, binding VEGF with high affinity. Cross-linking studies with radiolabelled VEGF showed complexes of size 115–145 and 220 kDa, interpreted to be one molecule of receptor with monomeric or dimeric VEGF, and VEGF dimer with two receptor molecules respectively. Truncated cDNA encoding two further potential soluble forms of Flt-1 have been found by RT-PCR in ovarian carcinoma cell lines, primary ascitic cells, chorion and HUVEC (Boocock *et al.*, 1995). Generated by insertion of 85 bp at position 1551 and 65 bp at position 1660 of the Flt-1 sequence numbered from the initiating ATG codon, both insertions contain in-frame translation termination codons, thus encoding variants which include the first five Ig-like domains and terminate in either 24 or 14 unrelated amino acids. The 150 kDa form of Flt-1 identified in our study cannot be any of these previously described forms on the basis of size, and additionally the protein sequence data obtained in our study confirm the presence of an amino acid sequence lying 3' to the termination region of these latter two variants.

The additional broad band at 220–250 kDa seen after electrophoresis of the VEGF-adsorbed amniotic fluid under non-reducing conditions shows a peptide mass fingerprint similar to that of the 150 kDa protein and thus may be derived from the 150 kDa protein as a result of binding of VEGF or another protein. Alternatively it may be a VEGF-linked dimeric form of the soluble Flt-1 variant previously identified in the supernatant of HUVEC (Kendall *et al.*, 1996) and which may be the faint band seen at 100–110 kDa under reducing conditions. From a comparison of the results of the radiolabelled cross-linking experiments seen in our study with those of the earlier study we believe the latter possibility to be unlikely. Evaluation of the kinetics of the binding using the Biosensor was not performed as the protein was not purified to homogeneity and the contributions from other variant Flt-1 molecules if present could not be ruled out. Apart from the very faint band at 110 kDa in the affinity-purified preparations, we failed

to detect the presence of other soluble Flt-1 variants in subsequent purification steps, even though the soluble form found in HUVEC supernatant is known to bind heparin–Sephacrose (Kendall *et al.*, 1996). This may be due to differences in relative concentration or to their absence in amniotic fluid, as their presence in biological fluids *in vivo* has yet to be demonstrated.

Our finding of transcripts of 7.5, 3.4 and 2.6 kb, in placenta, amnion and chorion are in broad agreement with previous studies (Shibuya *et al.*, 1990; Barleon *et al.*, 1994; Barleon *et al.*, 1996), with the 3.4 kb band the dominant transcript. Similar transcripts have been found in monocytes and polymorphonuclear cells (PMN) (Barleon *et al.*, 1996). The 7.5 kb transcript corresponds to the mRNA for the whole molecule with the smaller possibly being transcripts for soluble variants (Shibuya *et al.*, 1990). Whether these code for any of the soluble variants described is not known although theoretically such variants could possibly have longer transcripts than the full-length molecule unless transcription is also terminated prematurely together with the translation. Our failure to detect any of these transcripts in the HUVEC samples must be due to lack of sensitivity, as the Flt-1 molecule and the main soluble variant previously described (Kendall and Thomas, 1993) were clearly present as indicated by RT-PCR. Similar negative or weak Northern results for HUVEC have been seen (Barleon *et al.*, 1994, 1996), although with human endothelial cells from various tissues a doublet at 7.5–8.0 kb was apparent (Barleon *et al.*, 1994).

RT-PCR indicated the presence of the soluble variant described in HUVEC (Kendall and Thomas, 1993) in all tissues examined and the finding of positive bands for the cytoplasmic portion of the molecule would similarly indicate the presence of the full-length molecule in these tissues. This is not unexpected given the previous findings of Flt-1 in placenta and related tissues. Similar findings in cord blood are also in agreement with previous findings of Flt-1 mRNA in monocytes, PMN, CD34+ cells, megakaryocytes and platelets (Katoh *et al.*, 1995; Barleon *et al.*, 1996). In contradiction to previous studies (Boocock *et al.*, 1995) we failed to demonstrate mRNA for the two smaller soluble variants in placenta and chorion samples but this may have been due to the greater sensitivity achieved in the earlier study with the use of nested primers. Using RT-PCR we were unable to detect the mRNA for the novel soluble Flt-1 variant described. Several reasons may account for this. For example, if the molecule is generated by a large insertion encoding a premature stop codon or by read-through into an intron, such a large mRNA species may not have been amplified under the conditions described here and may require primers specific to the 'unique' sequence. An alternative possibility is that the molecule may be fetally derived and therefore not expressed in the tissues we have examined. Although proteolytic cleavage can account for the presence of truncated molecules, such cleavage in the extracellular region of Flt-1 would generate a molecule of too small a size for that described here.

The regulation of the switching of transcription to these alternate forms is not yet clear. Although the inhibition of VEGF by soluble forms of Flt-1 may be achieved directly by

sequestration, an alternative possibility may be the formation of heterodimers with membrane-bound Flt-1 which prevents the assembly of functional membrane-bound dimers necessary for signal transduction. Additionally the soluble variant previously found (Kendall *et al.*, 1996) was able to form a dimeric complex with the extracellular region of KDR and VEGF, again potentially leading to an inhibition of signalling, this time via KDR in addition to Flt-1. As homodimeric PlGF also binds to Flt-1, although with lower affinity than VEGF (Park *et al.*, 1994; Sawano *et al.*, 1996), it is conceivable that the soluble Flt-1 molecules can also regulate PlGF bioavailability. Whether or not the protein described in this study is able to inhibit the biological activity of VEGF can be investigated when it is either purified to homogeneity or expressed as a recombinant molecule. Additionally the possibility can be explored that the expression of the binding protein is co-ordinately regulated with VEGF by factors known to affect VEGF expression, such as steroid hormones (Smith, 1996; Greb *et al.*, 1997).

Another important aspect that this work has illustrated is the potential inappropriateness of some immunoassays for measuring VEGF in pregnancy-related samples due to the masking of the molecule by 'binding proteins'. The degree of interference will depend on the assay design, therefore studies measuring VEGF in samples from pregnant women must evaluate the assays used for this possibility. Studies utilizing the commercially available assay used in this study (e.g. Lyall *et al.*, 1997) are invalid as the measurements obtained are partly determined by the amount of binding protein and hence free VEGF which can be detected by this assay. Similarly studies using serum rather than plasma for the measurement of VEGF in any condition (Kondo *et al.*, 1994; Baker *et al.*, 1995; Hanatani *et al.*, 1995; Ferrari and Scagliotti, 1996; Takano *et al.*, 1996; Watanabe *et al.*, 1996; Lyall *et al.*, 1997) are also flawed as platelets contain VEGF which is released upon clotting, and hence serum concentrations are partly dependent on platelet number and platelet VEGF content (Banks *et al.*, 1997).

Clearly a naturally occurring protein with VEGF-binding activity is of great interest, both in the elucidation of the normal and pathogenic regulation of the angiogenic and trophoblastic regulatory activity of VEGF and because of its potential therapeutic applications both in pregnancy-related disorders and also in clinical situations with undesirable angiogenesis including cancer and diabetic retinopathies. Further studies to elucidate the exact structure of this variant should include extensive probing of cDNA libraries of fetal and placental tissue and large-scale purification of more material to gain further protein sequence data.

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## Detection and quantification of complexed and free soluble human vascular endothelial growth factor receptor-1 (sVEGFR-1) by ELISA

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### Abstract

Vascular endothelial growth factor (VEGF) is an important factor for endothelial cell proliferation and a key regulator of blood vessel development in embryos and angiogenesis in adult tissues. Its biological activity is mediated by two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR). In contrast to VEGFR-2, a naturally occurring soluble form of the VEGFR-1 (sVEGFR-1) is produced by endothelial cells by differential splicing of the *flt-1* gene, and it is a secreted gene product. In order to develop a specific enzyme-linked immunosorbent assay (ELISA) for the measurement of sVEGFR-1, we established five anti-human receptor antibodies and characterized them in detail. These antibodies recognize different epitopes located within the seven Ig-like domains of the extracellular receptor protein but have no neutralizing activity in ligand binding assays. Together with a polyclonal antiserum, a specific human sVEGFR-1 ELISA was developed using the mAb #190.11. The ELISA can detect human sVEGFR-1 with a minimum detection limit of 1 ng/ml. The ELISA does not show any cross-reactivity with other related soluble receptors. Using this assay, human sVEGFR-1 was measured in the supernatant of different VEGFR-1 expressing cell types. No sVEGFR-1 protein was detectable after heparin Sepharose treatment or size-exclusion filtration (< 30 kDa). The ELISA assay for sVEGFR-1 was also used to measure the amount of the soluble receptor in amniotic fluid samples of patients undergoing amniocentesis during the course of normal pregnancies. The concentration of the samples was in the range of 5–35 ng/ml. This ELISA could be useful powerful tool for investigations concerning the physiological function of the soluble receptor under normal and pathophysiological conditions.

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**Abbreviations:** VEGF, vascular endothelial growth factor; sVEGFR-1, soluble vascular endothelial growth factor receptor 1; KDR, kinase domain region receptor; Flt-1, *fms*-like tyrosine kinase 1; Flt-4, *fms*-like tyrosine kinase 4; PDGF-R $\beta$ , platelet-derived growth factor-receptor beta; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PlGF, placenta growth factor; mAb, monoclonal antibody; rh, recombinant human;  $M_r$ , relative molecular weight; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; FCS, fetal calf serum; MVEC, microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell

Furthermore, it may facilitate studies of the mechanisms of receptor production. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** VEGF; Soluble VEGFR-1; ELISA; Angiogenesis

## 1. Introduction

Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and angiogenesis that acts specifically on endothelial cells, because the VEGF receptors are almost exclusively expressed by the endothelium (Risau, 1997). As VEGF has the potency to increase vascular permeability, it has also been termed as vascular permeability factor (VPF). The *fms*-like tyrosine kinase (Flt-1, VEGFR-1) is a receptor for VEGF-A, VEGF-B and placenta growth factor (PlGF) (Olofsson et al., 1998). The gene for VEGFR-1 was first described by Shibuya et al. (1990). It is almost exclusively expressed on endothelial cells, but is also found on monocytes, where it is thought to mediate chemotaxis and tissue factor activation in response to VEGF (Clauss et al., 1996). Another receptor for VEGF is the human kinase-domain-region/mouse fetal-liver-kinase-1 (KDR/Flk-1, VEGFR-2) receptor. KDR binds VEGF-A, VEGF-C and VEGF-D (Achen et al., 1998) and is not found on monocytes. VEGFR-1 ( $K_d$  of 10–12 pM) has a higher affinity for rhVEGF<sub>165</sub> than VEGFR-2 ( $K_d$  of 75–125 pM). Both receptors share common features such as seven Ig-like extracellular domains, a single transmembrane region, and a consensus tyrosine kinase sequence interrupted by a kinase insert domain. Their molecular weight is about 220 kDa, and they are highly glycosylated. VEGFR-1, with its seven Ig-like extracellular domains, is structurally closely related to the PDGF receptor with five Ig-like extracellular domains and also to the receptors of the CSF-family (Kondo et al., 1998). Whereas for KDR, no naturally occurring soluble forms have been reported, the existence of natural soluble forms of VEGFR-1 (sVEGFR-1) was predicted by Shibuya et al. (1990), and later found as a cDNA from a human umbilical vein endothelial cell (HUVEC) cDNA library (Kendall and Thomas, 1993). The soluble VEGFR-1 is not generated by proteolysis on the cell surface like other cytokine

receptors. It is an alternatively spliced variant of the *flt-1* gene containing a unique C-terminal extension of 31 residues derived from an intron-sequence and has a molecular weight of ~110 (Kendall and Thomas, 1993). The amount of sVEGFR-1 secreted by cells has not previously been determined. A high level of sVEGFR-1 has been predicted to occur in sera and amniotic fluids of women as a pregnancy-associated factor following biochemical studies and isolation of the protein (Banks et al., 1998). In this paper, we report for the first time the development of a quantitative enzyme-linked immunosorbent assay (ELISA) for sVEGFR-1, which recognizes both free and complexed forms of sVEGFR-1. To the best of our knowledge, we present the first quantitative data for sVEGFR-1 in human biological fluids and cell culture supernatants.

## 2. Materials and methods

### 2.1. Generation of serum-free cell culture supernatants

Primary human dermal microvascular endothelial cells (MVEC; Bio Whittaker, MD, USA) and primary HUVEC (Bio Whittaker, MD, USA) were cultured in EBM medium (Bio Whittaker, MD, USA) containing 5% FCS and growth factor supplements according to the manufacturer's instructions. The COLO-800 and Mel-HO melanoma cell lines (both DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany) containing 5% FCS. All cells were grown at 37°C and 5% CO<sub>2</sub>. When the monolayers had reached 80% confluency, the cells were washed twice with PBS and afterwards incubated in serum-free media (Gibco BRL, Karlsruhe, Germany). After 2 days, the supernatants were harvested, centrifuged for 10 min at 1500 × *g* and immediately frozen at –20°C. For

immunoblotting, serum-free COLO-800 tissue culture supernatants were adjusted to 0.5 M NaCl and passed twice over a 1-ml HiTrap Heparin Sepharose column (Pharmacia-Biotech, Uppsala, Sweden). After elution with 1.5 M NaCl, the first 2 ml fraction was incubated with 10% (v/v) trichloroacetic acid for 0.5 h on ice followed by centrifugation for 15 min at  $20\,000 \times g$  and  $4^{\circ}\text{C}$ . The pellet was washed three times in 100% ethanol and, after air-drying, dissolved in 20  $\mu\text{l}$  water.

## 2.2. Collection of amniotic fluids and sample preparation

Amniotic fluids were collected from women with normal pregnancies undergoing amniocentesis. Only leftovers of diagnostic samples were used for the present study. Samples were centrifuged for 10 min at  $20\,000 \times g$  and the supernatant was frozen at  $-20^{\circ}\text{C}$ . For the quantitative ELISA measurement, the samples were desalted on NAP-5 columns (Pharmacia-Biotech, Uppsala, Sweden) and eluted with PBS directly before starting the ELISA. Soluble VEGFR-1 was removed from samples either by passing the amniotic fluids over a 1-ml HiTrap Heparin Sepharose column (Pharmacia-Biotech, Uppsala, Sweden) or by size exclusion filtration  $< 30$  kDa (Amicon, Beverly, MA, USA).

## 2.3. Proteins and antibodies

The recombinant human proteins sVEGFR-1<sub>D1-5</sub>, sVEGFR-1<sub>D1-6</sub> and VEGF<sub>165</sub> were produced in insect cells and purified from supernatants as previously described (Röckl et al., 1998). Soluble VEGFR-3 was produced and purified as described by Joukov et al. (1996). The polyclonal antibody against sVEGFR-1 was produced by a similar procedure to that described for the polyclonal sVEGFR-2 antibody (Röckl et al., 1998). Briefly, a total amount of 380  $\mu\text{g}$  sVEGFR-1 (D1–D5) was used for immunization of a New Zealand white rabbit. Total IgG from rabbit serum was isolated using HiTrap Protein A Sepharose columns (Pharmacia-Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The generation of mouse monoclonal anti-VEGFR-1 antibodies has been described in detail by Simon et al. (1998).

## 2.4. Immunoblotting

All samples were loaded onto a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated under reducing conditions. Immunoblotting onto a PVDF membrane (Millipore, Bedford, USA) was performed for 20 min at 15 V in a semi-dry blotting chamber (Biorad, München, Germany). The membrane was saturated with 20% non-fat milk in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 1 h at room temperature. mAb #190.11 was used diluted 1/200 in TBS containing 10% non-fat milk for 1 h at room temperature. After three washes for 5 min with TBST (TBS, 0.1% TWEEN 20), a secondary goat-anti-mouse alkaline phosphatase-conjugated antibody (Promega, Madison, USA) was used diluted 1/7500 in TBS containing 10% non-fat milk for 1 h at RT. After three washes for 5 min in TBST, the membrane was incubated in 10 ml Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, USA) for colour development.

## 2.5. Soluble VEGFR-1 ELISA

The monoclonal antibody #190.11 was coated to 96-well flat-bottom maxisorp plates (Nunc, Wiesbaden, Germany) by incubating all wells with 100  $\mu\text{l}$  ascitic fluid diluted 1/100 in PBS (pH 7.4) at  $4^{\circ}\text{C}$  overnight. After washing each well twice with 250  $\mu\text{l}$  washing buffer (50 mM Tris, 0.2% TWEEN 20, pH 8.0), the wells were saturated for 1 h at room temperature using 250  $\mu\text{l}$ /well PBS, pH 7.4 containing 1% FCS. All subsequent incubation steps were carried out at room temperature on an orbital shaker with three washes between the incubation steps. The volume of solutions added to the wells was always 100  $\mu\text{l}$ . Standard sVEGFR-1 and samples were diluted in assay buffer (PBS, pH 7.4 containing 1% FCS) and incubated for 1 h. Afterwards, the polyclonal detection antibody was used diluted to 1  $\mu\text{g}/\text{ml}$  IgG in assay buffer for 1 h, followed by incubation with biotinylated anti-rabbit-IgG (Dianova, Hamburg, Germany) diluted 1:50 000 in assay buffer for 1 h and incubation with Streptavidin-enzyme conjugate (Endogen, Woburn, MA, USA) diluted 1:20 000 in assay buffer for 0.5 h. After the

addition of TMB (tetra-methyl-benzidine; Boehringer Mannheim, Germany) substrate solution (0.1 M sodium-acetate, pH 5.5 containing 0.1 mg/ml TMB and 0.004%  $H_2O_2$ ) and stopping the reaction with 50  $\mu$ l/well 1 M  $H_2SO_4$ , the absorbance was measured at 450 nm and 650 nm (reference) in a microplate reader (Biorad, München, Germany). Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicate.

### 3. Results

#### 3.1. Characterization of anti human sVEGFR-1 mAbs

Based on our previous results from screening assays with a conventional ELISA procedure (Simon et al., 1998), five mouse hybridomas producing anti human sVEGFR-1 were selected: mAbs #64.2, #114.3, #190.11, #319.12 and #352.1. Table 1 summarizes the results of their characterization. The clones #64.2 and #319.12 have a weaker reactivity with sVEGFR-1 in ELISA than the other clones. All five monoclonals belong to the IgG 1 subclass and none of these clones had neutralizing activity as evaluated by a binding assay with iodinated VEGF<sub>165</sub>. The five mAbs were characterized for their minimum binding domain by immunoblotting with different truncated sVEGFR-1 constructs as depicted in Table 1. Clones #114.3 and #190.11 recognized the smallest derivatives of sVEGFR-1, suggesting that the binding-epitopes of these two antibodies were

Table 1  
Characterization of anti-human s VEGFR-1 mAbs

Clone	Isotype	ELISA	Inhibition activity	Immunoblot	Minimum binding domain
#352.1	IgG1	++	–	++	D1–D4 <sup>a</sup>
#114.3	IgG1	++	–	++	D1–D2
#319.12	IgG1	+	–	+	D1–D4
#190.11	IgG1	++	–	+	D1–D2
#64.2	IgG1	+	–	+	D1–D5

<sup>a</sup>The minimum binding domain is defined as the number of extracellular IgG-like loops of the VEGFR-1 necessary to display a positive reaction on immunoblotting scores: (–) not active; (+) intermediate activity; (++) strong activity.

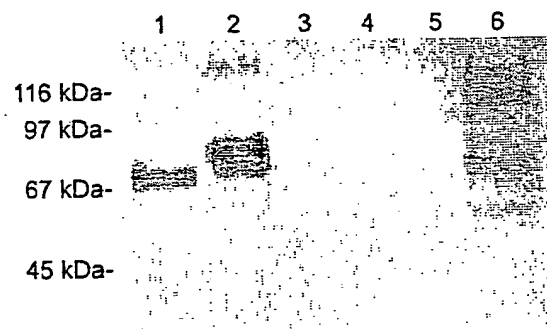


Fig. 1. Immunoblot with the soluble VEGFR-1 and related soluble receptors using the monoclonal antibody 190.11. Proteins were separated by 10% SDS-PAGE and blotted on to PVDF membranes as described in Section 2. After saturation, the membrane was incubated for 1 h with mAb #190.11 diluted 1/200 in TBS containing 10% non-fat milk followed by incubation with a goat-anti-mouse alkaline phosphatase-conjugated secondary antibody. Lane 1: 250 ng sVEGFR-1<sub>D1–5</sub>; lane 2: 250 ng sVEGFR-1<sub>D1–6</sub>; lane 3: 250 ng sVEGFR-2<sub>D1–7</sub>; lane 4: 250 ng sVEGFR-3; lane 5: 250 ng PDGFR- $\beta$ ; lane 6: sVEGFR-1 from 10 ml serum-free COLO-800 tissue culture supernatant after heparin Sepharose concentration.

located on the extracellular immunoglobulin-like domains 1 or 2. The antibody #190.11 was also used for immunoblot analysis with related soluble receptors. Moreover, the antibody did not show any cross-reactivity with the two other VEGF receptors or with PDGFR- $\beta$  (Fig. 1). Furthermore, the antibody recognized natural occurring sVEGFR-1 from COLO-800 cells, which is secreted as two different bands between 110 and 130 kDa.

#### 3.2. Detection of ligand-complexed and free sVEGFR-1

Among the five mAbs, clone #190.11, with its binding-epitope located within the first two extracellular domains of sVEGFR-1, was chosen as capture antibody. A polyclonal serum was used as the detector. The combination of these two antibodies resulted in the most sensitive ELISA. An increasing amount of recombinant human VEGF<sub>165</sub> was added in order to cause complex formation between sVEGFR-1 and its ligand. As shown in Fig. 2, a 50-fold excess of VEGF<sub>165</sub> did not interfere with the binding of 10 ng/ml sVEGFR-1 to the capture antibody. The absorbance value obtained was similar to that given by

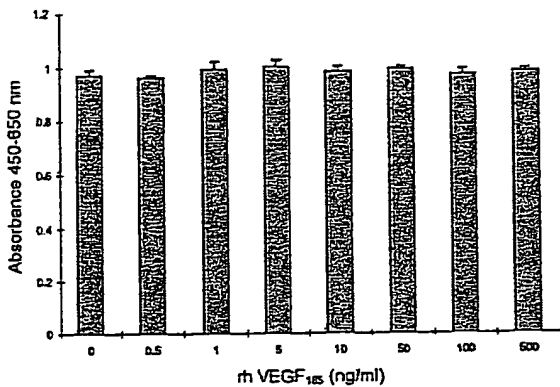


Fig. 2. Monoclonal antibody #190.11 binds to human sVEGFR-1 independently of ligand receptor complex formation. Soluble VEGFR-1 (10 ng/ml) was added to each well coated with mAb #190.11 in the absence or presence of increasing concentrations of human VEGF<sub>165</sub>. The polyclonal rabbit anti-VEGFR-1 antibody followed by a biotinylated goat anti-rabbit antibody was used to detect and quantify bound sVEGFR-1. All measurements were performed in duplicate. Results show the mean absorbance at 450 nm + ISD.

10 ng/ml sVEGFR-1 without addition of the ligand, indicating an equal perception of ligand-complexed and free soluble receptor by the capture antibody.

### 3.3. Standard curve and cross-reactivity

A typical standard curve obtained with the ELISA is shown in Fig. 3. The minimum detection limit estimated by serial dilution was 1 ng/ml recombinant human sVEGFR-1. To exclude possible cross-reactions with other soluble receptor-proteins, which are closely related to the extracellular part of VEGFR-1, the ELISA was evaluated for reactivity with sVEGFR-2, sVEGFR-3 and soluble platelet-derived growth factor receptor beta (sPDGFR- $\beta$ ). All proteins were diluted in the same serial dilution as sVEGFR-1. The assay showed no cross-reactivity with these soluble receptors even at concentrations 75-fold above the detection limit (Fig. 3).

### 3.4. Influence of pre-treatment on detection of VEGFR-1

It has previously been reported that sVEGFR-1 is present in conditioned media of HUVECs (Kendall et al., 1996; Barleon et al., 1997). This heparin

binding protein (Röckl et al., 1998) is found in amniotic fluids as well and has a molecular weight of 150 kDa when monomeric and 400–500 kDa when multimeric (Banks et al., 1998). Amniotic fluids and HUVEC-conditioned media were analyzed without pre-treatment, after passing over a heparin Sepharose column and after size exclusion filtration through a 30-kDa cut-off membrane (Fig. 4). Centrifuged samples of untreated, amniotic fluids (14th week of gestation) contained about 17 ng/ml sVEGFR-1, while HUVEC-conditioned media contained about 10 ng/ml sVEGFR-1. After either pre-treatment procedure described above, the amount of sVEGFR-1 was significantly reduced in both samples. This indicates that the ELISA is specific for sVEGFR-1 and that removal of the soluble receptor from biological fluids and cell supernatants does not result in cross-reactivity with other proteins.

### 3.5. Intra- and inter-assay variation

To estimate the precision and the reproducibility of the ELISA, intra- and inter-assay variations were evaluated. Amniotic fluid samples, collected from normal pregnancies during amniocentesis and de-

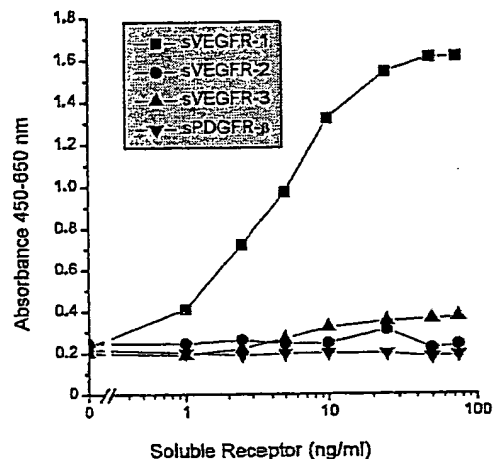


Fig. 3. Human sVEGFR-1 standard curve and cross-reactivity with related soluble growth factor receptors. The extracellular domains of the receptors for VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR- $\beta$  were diluted in assay buffer up to 75 ng/ml and a typical standard curve was measured according to the ELISA procedure described. All measurements were performed in duplicate. The results show the absorbance at 450 nm as mean values.

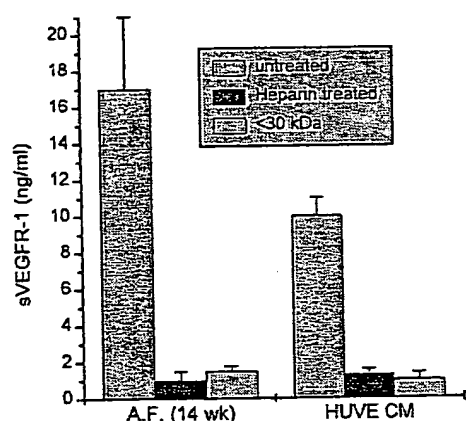


Fig. 4. Quantification of sVEGFR-1 in amniotic fluid (AF) and human umbilical vein endothelial cell conditioned media (HUVEC CM) by standard ELISA after treatment with heparin Sepharose and after filtration with a 30-kDa cut-off membrane. All measurements were performed in duplicate and the results shown are means + 1SD.

pleted of sVEGFR-1 by size exclusion filtration through a < 30-kDa cut-off membrane, were spiked with recombinant human sVEGFR-1 to final concentrations of 5, 15 and 30 ng/ml, respectively. Intra-assay variations were estimated from six wells in the same plate, inter-assay variations were calculated from duplicate measurements in four independent experiments. Both, the intra- and inter-assay coefficients of variation (CVs) were below 10% (Table 2),

Table 2  
Intra- and inter-assay variations in amniotic fluid samples

Sample	n	Mean (ng/ml)	SD	CV (%)
<i>Intra-assay variation</i>				
1	6	4.5	0.26	5.77
2	6	16.1	1.43	8.88
3	6	28.0	2.24	8.00
Mean				7.55
<i>Inter-assay variation</i>				
1	4	4.3	0.23	5.35
2	4	15.8	1.09	6.89
3	4	29.0	2.64	9.10
Mean				7.11

Amniotic fluid samples collected from normal pregnancies were depleted of sVEGFR-1 by size exclusion filtration < 30 kDa and spiked with 5 (1), 15 (2) and 30 (3) ng/ml recombinant sVEGFR-1.

demonstrating the reproducibility and reliability of the assay.

### 3.6. Quantification of sVEGFR-1 in cell culture supernatants

It has been reported that the *flt-1* gene codes for the transmembrane receptor as well as for a soluble receptor generated by differential splicing (Kendall and Thomas, 1993). The exact data on the expression of sVEGFR-1 by different cell types remain unclear. To verify the efficiency of the ELISA in measuring sVEGFR-1 in cell culture supernatants, we chose a variety of serum-free media conditioned for 48 h (Table 3). The primary cell types MVEC and HUVEC secreted sVEGFR-1 in almost equal amounts of about 10 ng/ml. Soluble VEGFR-1 was not detectable in primary monocyte-conditioned media and Mel-HO (melanoma cell line)-conditioned media. The supernatants of monocytes and Mel-HO cells were also negative, when they were subjected to SDS-PAGE and immunoblotting after concentration by incubation with heparin Sepharose (data not shown). The second melanoma cell line COLO-800, which expresses VEGFR-1 as detected by Northern blotting analysis (data not shown), secreted sVEGFR-1 at 12 ng/ml over an incubation period of 48 h in serum-free media.

### 3.7. Quantification of sVEGFR-1 in amniotic fluids

Amniotic fluids obtained from amniocentesis of normal pregnancies showed a broad range of

Table 3  
Detection of s VEGFR-1 in tissue culture samples

Sample	n	Mean (ng/ml)	SD
HUVEC	3	10	1.41
MVEC	3	9	2.05
Mel-HO	3	0	–
COLO-800	4	12	3.10
Monocytes	3	0	–

Samples were prepared from serum-free media conditioned for 48 h by HUVEC, MVEC, a melanoma cell line (Mel-HO), a colon cancer cell line (COLO-800) and human peripheral blood monocytes. The cells were grown to 80% confluency before addition of serum-free media.

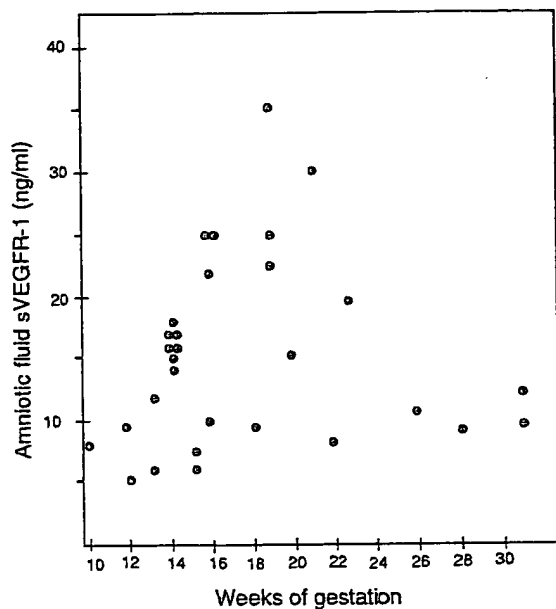


Fig. 5. Soluble VEGFR-1 amniotic fluid levels from pregnant women undergoing amniocentesis at different weeks of gestation. All measurements were performed in duplicate using the ELISA procedure described. Results are shown in nanogram per milliliter sVEGFR-1.

sVEGFR-1 content. Fig. 5 shows the sVEGFR-1 concentration of amniotic fluids in relation to the weeks of gestation. The amount of sVEGFR-1 in amniotic fluids ranged from 5 to 35 ng/ml. Samples taken at an earlier or later time point, e.g., around week 10 or week 30, appear to have a lower content of sVEGFR-1 than samples obtained around week 20. Many more samples taken at every time point will be necessary in order to investigate possible variations during pregnancies. However, the results are in good agreement with earlier reported findings that amniotic fluids may have sVEGFR-1 concentrated up to 25–50 ng/ml (Banks et al., 1998).

#### 4. Discussion

The present study describes for the first time the establishment of a quantitative ELISA for soluble VEGFR-1. Five monoclonal antibodies against recombinant human extracellular VEGFR-1 were generated, characterized and tested for their usefulness to detect sVEGFR-1. The monoclonal antibodies rec-

ognize different Ig-like domains of the extracellular part of the receptor. However, none of the monoclonal antibodies had neutralizing activity when tested in a solid phase assay with iodinated VEGF and bound VEGFR-1. In combination with a polyclonal serum generated against the recombinant protein, the mAb #190.11 was used to develop a sensitive and quantitative ELISA. The ELISA detects complexed and free human sVEGFR-1 with a minimum detection limit of 1 ng/ml and does not cross-react with other related soluble receptors, such as sVEGFR-2, sVEGFR-3 and sPDGFR- $\beta$ . This observation is important, since all four receptors share a high degree of homology which is of the order of 30% (Barleon et al., 1994). The ELISA does not react with amniotic fluid samples from which sVEGFR-1 has been removed by heparin Sepharose treatment or by size exclusion filtration. So far, the presence of sVEGFR-1 has only been detected by complex formation with iodinated VEGF or by biochemical purification methods (Banks et al., 1998). Therefore, the ELISA is very useful for measurements of the total amount of human sVEGFR-1 in a variety of clinical samples and in cell culture media. Moreover, the ELISA is much easier and faster than other assays, which use iodinated VEGF or large amounts of sample volumes of amniotic fluids for protein purification and quantification.

Taking advantage of the ELISA system, we measured sVEGFR-1 in cell culture supernatants and in amniotic fluid samples from patients undergoing amniocentesis. These are the first quantitative measurements of sVEGFR-1 in human biological fluids. Amniotic fluid samples from pregnancies between the 10th and 31st week of gestation contained concentrations of sVEGFR-1 between 5 and 35 ng/ml. Although the samples showed a large variation in the amount of sVEGFR-1, there was no obvious correlation with the gestational age of the patients. These data are in good agreement with other reports on the presence of sVEGFR-1 as a pregnancy-associated protein (Banks et al., 1998). It was estimated from their protein purification studies that the initial concentration of the soluble receptor in pooled amniotic fluid samples may be in the range of 25–50 ng/ml. The soluble receptor protein was originally cloned as a splice variant of the *flt-1* gene and later detected in the supernatant of human vascular endothelial cells

(Kendall and Thomas, 1993; Kendall et al., 1996). Here, we were able to show that sVEGFR-1 can be detected and quantified in conditioned media from endothelial cells and in one melanoma cell line, where *flt-1* mRNA expression has previously been reported (Terman et al., 1994). However, different concentrations can be detected in endothelial cell types and melanoma cell types, when conditioned media are used after 48 h of pre-incubation. Other soluble receptors have been reported for FGF (Hanneken et al., 1994) or IL-4 (Novick et al., 1989) and various other cytokines (Fernandez Botran and Vitetta, 1991).

The physiological role of sVEGFR-1 is not known, but it is likely to be a negative regulator of VEGF availability, or it may prolong the different VEGF activities associated with this protein. By analogy with other soluble receptors and their ligands, VEGF complexed to a soluble VEGF receptor may be protected from proteases resulting in a longer half-life for VEGF. Endogenous sVEGFR-1 may act as a decoy receptor for VEGF regulating the bioavailability of VEGF by sequestering VEGF in the endothelial microenvironment and may therefore influence the angiogenic balance. However, the ratio of free to receptor complexed VEGF may determine its net biological activity. Also, sVEGFR-1 could form heterodimers with membrane-bound VEGFR-1 and -2 and abolish their signal transduction by acting as a dominant negative inhibitor. Thus, the human sVEGFR-1 ELISA could become a powerful tool for investigations and clarifying the roles of the soluble receptor during angiogenic processes in health and disease. Another possible application would be the analysis of regulatory mechanisms of sVEGFR-1 production by reliable determination of the ligand in a variety of cell culture samples and clinical relevant samples from patients having angiogenic diseases or undergoing anti-angiogenic therapies.

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## Vascular endothelial growth factor is bound in amniotic fluid and maternal serum

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To study vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) concentrations and their possible binders, serum from 22 non-pregnant and 55 pregnant women (15 at weeks 10–13; 40 at term), umbilical vein ( $n = 24$ ) and artery ( $n = 13$ ) and amniotic fluid (a pool of 50 at weeks 15–17; 11 at term) were assessed for VEGF and PlGF by an enzyme-linked immunosorbent assay. In amniotic fluid and maternal serum VEGF concentrations were  $<16$  ng/ml and added VEGF was not recovered. VEGF was detected in serum from mothers post-partum ( $137 \pm 142$  ng/l, mean  $\pm$  SD), umbilical artery ( $421 \pm 288$  ng/l) and vein ( $502 \pm 339$  ng/l) and non-pregnant controls ( $182 \pm 147$  ng/l), and added VEGF was fully recovered. PlGF was detected in pregnancy serum ( $52 \pm 23$  ng/l early pregnancy;  $439 \pm 217$  ng/l term pregnancy) and in amniotic fluid (early pregnancy 56 ng/l; term pregnancy  $30 \pm 18$  ng/l). PlGF was fully recovered in all samples. Gel filtration and isoelectric focusing revealed that in maternal serum and amniotic fluid [<sup>125</sup>I]VEGF was bound to a protein with an  $M_r$  of 400–700 kDa and an isoelectric point of approximately 8. This protein was not identical with alpha-2-macroglobulin (by an immunofluorometric assay), pregnancy zone protein or pregnancy associated plasma protein-A (by immunodiffusion). In conclusion, VEGF-binding activity is present in amniotic fluid and maternal blood. It disappears after delivery and is not detectable in fetal or non-pregnant serum.

**Key words:** amniotic fluid/PlGF/pregnancy-associated binding/VEGF

### Introduction

The vascular endothelial growth factor (VEGF) family comprises VEGF (Gospodarowicz *et al.*, 1989; Keck *et al.*, 1989; Leung *et al.*, 1989), VEGF-B (Olofsson *et al.*, 1996), VEGF-C (Joukov *et al.*, 1996), VEGF-D (Orlandini *et al.*, 1996; Yamada *et al.*, 1997) and placenta growth factor (PlGF) (Maglione *et al.*, 1991). Of these secreted factors, VEGF is the most potent direct stimulator of vascular endothelial cell

growth. VEGF also induces vascular permeability (Keck *et al.*, 1989) and its expression is enhanced by hypoxia (Ladoux and Frelin, 1993). VEGF is known to be expressed in the placenta (Sharkey *et al.*, 1993; Vuorela *et al.*, 1997) and in several fetal tissues (Kaipainen *et al.*, 1993).

Two specific endothelial cell receptors, VEGF receptor-1 (VEGFR-1) or fms-like tyrosine kinase-1 (flt-1) (de Vries *et al.*, 1992) and VEGFR-2 or kinase insert domain-containing receptor (KDR) (Terman *et al.*, 1994), are known to bind VEGF, and a soluble form of VEGFR-1 has been identified (Kendall and Thomas, 1993; Kendall *et al.*, 1996). VEGFR-1 also binds PlGF with high affinity (Park *et al.*, 1994). Serum VEGF concentrations have earlier been reported to rise during the first trimester of pregnancy (Evans *et al.*, 1998), but it is not known to what extent the VEGF receptors or other possible factors regulate the bio-availability of VEGF and PlGF during early or later pregnancy.

The presence of some, so far unknown, gestational factors regulating angiogenesis seems obvious considering the strong local neovascularization occurring in the placenta and the placental bed. The original aim of this study was to measure VEGF and PlGF in amniotic fluid and serum during pregnancy, and to identify possible factors regulating their activity.

### Materials and methods

#### Subjects and sample collection

##### Pregnant subjects

After approval by the local ethics committee and informed consent, 40 healthy non-smoking women (median age 32 years, range 18–42 years) with uncomplicated pregnancies had venous blood samples drawn at gestational weeks 38–40, within the last 24 h before delivery, and on the third post-partum day ( $n = 21$ ). Immediately after delivery umbilical cord venous ( $n = 24$ ) and arterial ( $n = 13$ ) samples were collected. Maternal venous blood samples of early pregnancy (weeks 10–13) were collected from 15 healthy women (median age 29 years, range 21–37 years). All pregnant subjects had uncomplicated singleton pregnancies and delivered healthy newborns.

##### Amniotic fluid

A pool of 50 random amniotic fluid samples from gestational weeks 15–17 was prepared from samples submitted for assessment of fetal chromosomal abnormalities and assay of  $\alpha$ -fetoprotein (AFP). Eleven additional individual samples of amniotic fluid were collected during Caesarean section of healthy mothers at term. Samples were stored at  $-20^\circ\text{C}$  until analysis.

##### Non-pregnant subjects

Single blood samples were collected from 22 healthy non-pregnant, non-medicated and non-smoking women (median age 29 years, range 17–39 years). As serum VEGF concentrations have been shown to correlate with serum progesterone concentrations (Evans *et al.*, 1998),

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all the samples were collected at the same phase of the menstrual cycle, i.e. the follicular phase.

Although no diurnal variation of urinary VEGF concentrations has been observed in gonadotrophin-treated women (Robertson *et al.*, 1995), all blood samples were collected between 8 and 9 a.m. Following separation, serum was frozen and stored at  $-20^{\circ}\text{C}$  until analysis.

#### Immunassays of VEGF and PlGF

VEGF and PlGF were quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions [Quantikine recombinant human (rh)VEGF and Quantikine rhPlGF, R&D Systems Europe Ltd, Abingdon, UK]. The detection limit of the assays was 16 ng/l. Recovery of rhVEGF and rhPlGF was tested by the ability of the ELISA to detect added rhVEGF (200 ng/l) or rhPlGF (100 ng/l) (proteins were provided as standard proteins in the ELISA) in various samples. The inter- and intra-assay coefficients of variation were 6.5 and 8.5% (Quantikine rhVEGF) and 6.3 and 7.9% (Quantikine rhPlGF). Recovery of added VEGF was also studied in serial dilutions of amniotic fluid and maternal serum samples from early and term pregnancy. According to the manufacturer, rhVEGF and rhPlGF ELISA show ~20% and 5% cross-reactivity respectively, with the naturally occurring PlGF/VEGF heterodimer (DiSalvo *et al.*, 1995). No cross-reactivity of either ELISA was observed with the novel growth factors VEGF-B (Olofsson *et al.*, 1996) and VEGF-C (Joukov *et al.*, 1996) when tested at concentrations 0.5–750 ng/l.

The possible influence of heparin on the ability of amniotic fluid to inhibit VEGF immunoreactivity in ELISA was studied by first incubating amniotic fluid with VEGF (see below) for 1 h at room temperature, after which heparin (0.05–5 mg/ml; Løvens Kemiske Fabrik, Ballerup, Denmark) was added to the samples and incubation was continued for 1 h at room temperature. VEGF concentrations were then measured by ELISA.

#### Immunodiffusion

The reactivity of the VEGF-binding factor with antibodies against  $\alpha_2$ -macroglobulin (anti- $\alpha_2\text{M}$ , 7.2 g/l; DAKO, Denmark), pregnancy zone protein (anti-PZ, 5.8 g/l; DAKO) and pregnancy-associated plasma protein-A (anti-PAPP-A, 4.2 g/l; DAKO) was studied by immunodiffusion in 1% agarose gels. Samples of amniotic fluid and serum from pregnant women at term and from non-pregnant women were studied, either directly or following 1 h incubation with [ $^{125}\text{I}$ ]VEGF at room temperature. A total of 10  $\mu\text{l}$  of antibody and sample were pipetted into the wells and incubated for 48 h at  $+4^{\circ}\text{C}$ . Gels were viewed for immunoprecipitates at 24 and 48 h, dried, and, after drying, subjected to autoradiography (X-Omat<sup>®</sup>; Eastman Kodak Company, Rochester, NY, USA).

#### Time-resolved immunofluorometric assay (IFMA)

The concentration of  $\alpha_2\text{M}$  in amniotic fluid was determined by an immunofluorometric method (IFMA) as described previously (Leinonen *et al.*, 1996). The detection limit of the assay was 5  $\mu\text{g/l}$ .

#### Radio-iodination of VEGF

Five  $\mu\text{g}$  of recombinant human VEGF<sub>165</sub> (Genzyme Diagnostics, Cambridge, MA, USA) was radiolabelled using Iodo Gen<sup>®</sup> (Pierce, Rockford, IL, USA). Sixty-one  $\mu\text{l}$  phosphate buffered saline (PBS; 0.14  $\mu\text{l}$  NaCl, 2.7  $\mu\text{l}$  KCl, 0.01  $\mu\text{l}$   $\text{Na}_2\text{HPO}_4$ , 1.76  $\mu\text{l}$   $\text{KH}_2\text{PO}_4$ , pH 7.4), 4  $\mu\text{l}$  [ $^{125}\text{I}$ ]Na (2 mCi) and 5  $\mu\text{l}$  of rhVEGF in 25  $\mu\text{l}$  of PBS were pipetted into a test tube coated with 200  $\mu\text{l}$  of dichloromethane containing 40 mg/l Iodo Gen<sup>®</sup>. After 15 min 10  $\mu\text{l}$  of 0.01  $\mu\text{l}$  KI was added. The labelled protein was separated from free iodine by gel filtration on a 1.5  $\times$  5 cm Sephadex<sup>®</sup> G-25 column (PD-10; Pharmacia

Fine Chemicals, Uppsala, Sweden) in PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA). Specific activity was 36 000 c.p.m./ng protein.

#### Gel filtration

A Sephacryl<sup>®</sup> S-300 HR (Pharmacia Biotech, Uppsala, Sweden) column (84  $\times$  1.5 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden) was equilibrated with tris-buffered saline (TBS; 0.05 mol/l Tris-HCl, 0.15 mol/l NaCl, pH 7.7). The exclusion volume of the column was 40 ml, flow rate was 15 ml/h (Microperpe<sup>®</sup> peristaltic pump, Pharmacia LKB Biotechnology) and 1 ml fractions were collected (Redifrac<sup>®</sup>, Pharmacia LKB Biotechnology). Thyroglobulin 669 kDa (Pharmacia Fine Chemicals), ferritin 440 kDa (Pharmacia Fine Chemicals), human immunoglobulin G (IgG) 168 kDa (Sigma Chemical Co.), bovine serum albumin 67 kDa (Sigma Chemical Co.), soybean trypsin inhibitor 20.1 kDa (Sigma Chemical Co.) were used as molecular weight markers.

Two ml of amniotic fluid pool was fractionated on the column. Five consecutive fractions were pooled, and recovery of VEGF in the pooled fractions was analysed by ELISA. Pooling of fractions was done to reduce expense. Despite this, it was found that the accuracy of the analysis still allowed verification of the VEGF binding activity eluting in fractions similar to those of the high molecular weight complexes observed in gel filtration analysis with [ $^{125}\text{I}$ ]VEGF. [ $^{125}\text{I}$ ]VEGF in 200  $\mu\text{l}$  TBS was loaded onto the column alone or after pre-incubation for 1 h at room temperature, with 200  $\mu\text{l}$  of amniotic fluid, serum from maternal, umbilical and non-pregnant samples. The fractions were monitored for absorbance at 280 nm (Lambda 3B UV/VIS Spectrophotometer<sup>®</sup>; Perkin-Elmer, Überlingen, Germany) and radioactivity (1260 Multigamma gamma counter<sup>®</sup>, LKB, Wallac, Sweden).

In another experiment 200  $\mu\text{l}$  of amniotic fluid and sera were separated on the column. Recombinant VEGF was added to the fractions (200 ng/l) and the recovery was measured by ELISA.

The effect of low pH and high salt concentration on the VEGF-binding complex was studied with a 50  $\times$  1 cm column packed with Sephacryl<sup>®</sup> S-300 HR and equilibrated with TBS. The flow rate was 15 ml/h and 0.5 ml fractions were collected. [ $^{125}\text{I}$ ]VEGF was incubated with amniotic fluid at room temperature for 1 h and loaded onto the column (200  $\mu\text{l}$ ) as such or following acidification to pH 2 with HCl for 15 min and neutralization with NaOH. Then [ $^{125}\text{I}$ ]VEGF incubated with amniotic fluid at room temperature for 1 h was separated at pH 2 without neutralization. In other experiments 200  $\mu\text{l}$  of [ $^{125}\text{I}$ ]VEGF pre-incubated with amniotic fluid for 1 h at room temperature was loaded onto the column which had been equilibrated with either 0.5, 1, 2, 3, 4 mol/l NaCl or with 4 mol/l KSCN.

#### Isoelectric focusing

NOVEX Precast Gel<sup>®</sup>, pH 3–10 (Novel Experimental Technology, San Diego, CA, US), was used for estimation of the isoelectric point of the complex studied according to the manufacturer's instructions. A total of 1 ng of [ $^{125}\text{I}$ ]VEGF in 4  $\mu\text{l}$  TBS-1%BSA was pipetted into the wells following 1 h incubation at room temperature with 9  $\mu\text{l}$  of either PBS, amniotic fluid or amniotic fluid with added 1 ng, 4 ng or 16 ng of unlabelled rhVEGF (Genzyme<sup>®</sup>), or the VEGF-binding fractions of amniotic fluid or pregnancy serum from early or term pregnancy. These VEGF-binding fractions were obtained from separation of samples by gel filtration in Sephacryl<sup>®</sup> S-300 HR columns, as described above.

#### Statistics

Between-group comparisons were performed using Student's unpaired *t*-test.

Table 1. The concentrations of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) in serum and amniotic fluid. Values are expressed as mean  $\pm$  SD

	VEGF (ng/l)	P value	PIGF (ng/l)	P value
<b>Serum</b>				
non-pregnant women (n = 22)	182 $\pm$ 147	<0.001 <sup>a</sup>	<16	<0.001 <sup>a</sup>
early pregnancy (n = 15)	<16	52 $\pm$ 23	<0.05 <sup>c</sup>	
term pregnancy (n = 40)	<16 ng/l	439 $\pm$ 217		
umbilical vein (n = 24)	502 $\pm$ 339	<0.001 <sup>b</sup>	<16	
umbilical artery (n = 13)	421 $\pm$ 288	<0.001 <sup>b</sup>	<16	
third post-partum day (n = 21)	137 $\pm$ 142	<0.001 <sup>c</sup>	21 $\pm$ 25	<0.001 <sup>a</sup>
<b>Amniotic fluid</b>				
early pregnancy (a pool of 50)	<16		56	
term pregnancy (n = 11)	<16		30 $\pm$ 18	<0.001 <sup>d</sup>

<sup>a,b,c,d</sup> Compared with pregnancy samples, maternal samples and term samples respectively.

<sup>c</sup> Compared with term serum.

## Results

Added VEGF was not recovered in serum samples of early and term pregnancy. However, full recovery of added VEGF was observed in post-partum maternal serum samples as well as in serum samples from umbilical artery and vein and from non-pregnant subjects. Added VEGF was not recovered in amniotic fluid from early or term pregnancy. However, full recovery of added PIGF was observed in all serum and amniotic fluid samples (Table 1). Gestational age, birth weight of child or placental weight did not correlate with either serum VEGF or PIGF concentrations (data not shown).

Upon dilution of the maternal serum samples and the amniotic fluid samples, the inhibitory effect on added VEGF decreased, and 50% of added rhVEGF was recovered at approximately 1:500 dilution of amniotic fluid from early pregnancy and 1:1000 dilution of amniotic fluid from term. In maternal serum samples the inhibitory effect on VEGF was weaker than in amniotic fluid, and 50% recovery of added rhVEGF was observed at approximately 1:10 dilution of serum from early and at 1:30 dilution of serum from term pregnancy (Figure 1).

In gel filtration (S-300 HR, 85  $\times$  1.5 cm column) [<sup>125</sup>I]VEGF eluted in fractions corresponding to an  $M_r$  of 40 kDa (Figure 2A). When [<sup>125</sup>I]VEGF was pre-incubated with amniotic fluid from early or term pregnancy, the major radioactive peaks (78 and 73% of total radioactivity in the sample respectively) eluted in fractions corresponding to an  $M_r$  of approximately 700 kDa. The remaining radioactivity eluted in fractions corresponding to an  $M_r$  of about 40 kDa (Figure 2A). Following pre-incubation of [<sup>125</sup>I]VEGF with serum from early or term pregnancy, the major radioactive peaks (67 and 61% of total radioactivity in the sample respectively) shifted to fractions corresponding to molecular weights of 700 kDa or more and 400 kDa respectively (Figure 2B). When untreated maternal serum was separated on the same column, the corresponding fractions were found to inhibit recovery of added VEGF.

When [<sup>125</sup>I]VEGF was pre-incubated with serum from umbilical venous or arterial blood or serum of non-pregnant subjects, only minor radioactive peaks (17, 18 and 35% of total radioactivity respectively) eluted in the high molecular weight fractions (Figure 2C). The pattern was similar to that

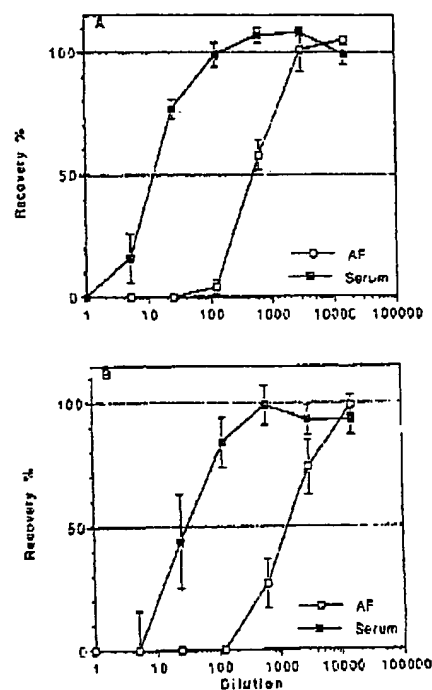


Figure 1. Vascular endothelial growth factor (VEGF)-binding activity is stronger in amniotic fluid than in maternal serum, as shown by recovery (%) of added recombinant human (rh)VEGF (200 ng/l) in serial dilutions of serum or amniotic fluid (AF) of early (A) and term (B) pregnancy.

of [<sup>125</sup>I]VEGF alone suggesting polymerization of part of rhVEGF as a result of iodination.

When [<sup>125</sup>I]VEGF was incubated with amniotic fluid and then separated by gel filtration on a column (S-300 HR, 50  $\times$  1 cm) equilibrated with 4 mol/l KSCN the elution profile was similar to that of [<sup>125</sup>I]VEGF alone. When [<sup>125</sup>I]VEGF incubated with amniotic fluid was separated in the same column using NaCl at concentrations of 0.5–4 mol/l in PBS, [<sup>125</sup>I]VEGF was not dissociated from the complex. Furthermore, the ability of amniotic fluid to shift the radioactive

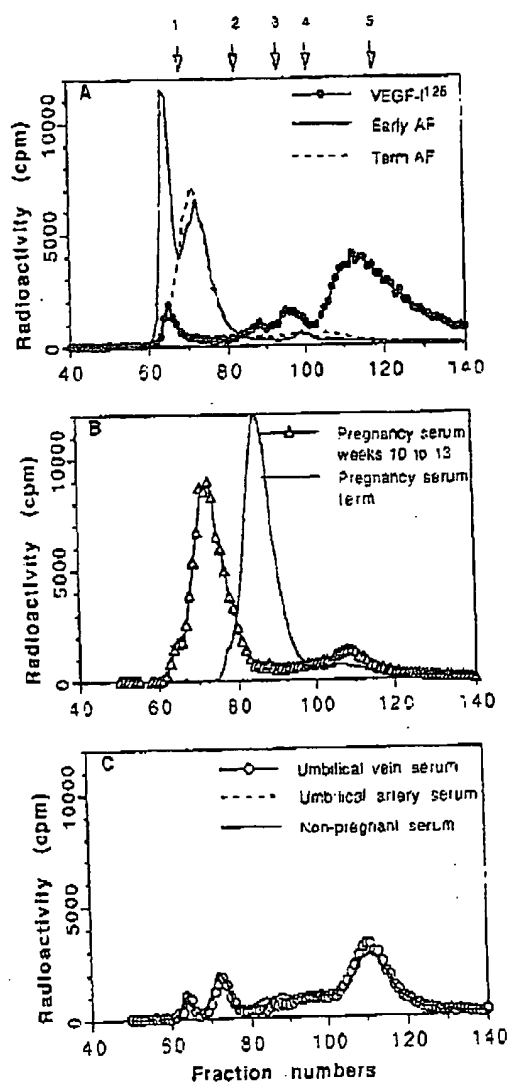


Figure 2. Sephadryl S-300 HR (85x1.5 cm) gel filtration analysis in tris-buffered saline showing binding of VEGF in amniotic fluid. A: [ $^{125}$ I]VEGF alone or following pre-incubation with amniotic fluid (AF) of early and term pregnancy. B: [ $^{125}$ I]VEGF following pre-incubation with serum (S) of early and term pregnancy. C: [ $^{125}$ I]VEGF following pre-incubation with serum from umbilical vein or artery from non-pregnant women. Arrows at top of figure indicate molecular weight markers: 1, thyroglobulin 669 kDa; 2, ferritin 440 kDa; 3, human immunoglobulin G 168 kDa; 4, bovine serum albumin 67 kDa; 5, soybean trypsin inhibitor 20.1 kDa.

peak to high molecular weight fractions was retained after acidification of the complex to pH 2 and neutralization. However, when the [ $^{125}$ I]VEGF complex was pre-incubated and separated at pH 2 without neutralization, no shift of the radioactive peak to the high molecular weight fractions was observed. Incubation with various concentrations of heparin before analysis in ELISA did not release VEGF from the complex in amniotic fluid.

In immunodiffusion with antibodies against  $\alpha$ 2M, PZ and

VEGF is bound during pregnancy

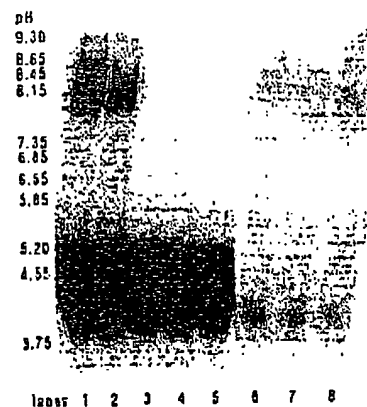


Figure 3. Isoelectric focusing of VEGF. The VEGF-binding complex of amniotic fluid is approximately at pH 8, and cold rhVEGF competes with [ $^{125}$ I]VEGF for the binding. [ $^{125}$ I]VEGF incubated with amniotic fluid (lane 1), or with amniotic fluid and equal (lane 2), 4-fold (lane 3) or 16-fold (lane 4) concentrations of rhVEGF as compared to [ $^{125}$ I]VEGF, or phosphate-buffered saline (lane 5), or VEGF-binding fractions obtained from Sephacryl<sup>®</sup> S-300 HR gel filtration of amniotic fluid (lane 6), serum from early (lane 7) or term (lane 8) pregnancy.

PAPP-A no precipitation lines with amniotic fluid were observed, whether studied alone or after pre-incubation with [ $^{125}$ I]VEGF. Serum of pregnant women at term showed precipitation lines with anti- $\alpha$ 2M and anti-PZ, but not with anti-PAPP-A. Serum from non-pregnant women showed precipitation lines with anti- $\alpha$ 2M, but not with anti-PAPP-A or anti-PZ. Autoradiography revealed bands corresponding to the precipitation lines for anti- $\alpha$ 2M, whereas no bands were seen with PZ or PAPP-A (data not shown). By IFMA no  $\alpha$ 2M was observed in amniotic fluid.

In isoelectric focusing [ $^{125}$ I]VEGF added to amniotic fluid, or to the VEGF-binding fractions of amniotic fluid or serum, displayed an isoelectric point of approximately 8. Addition of increasing amounts of non-radioactive rhVEGF caused dissociation of [ $^{125}$ I]VEGF, which then focused in the pH range 4-5 (Figure 3).

## Discussion

VEGF is a potent stimulator of vasculo- and angiogenesis. The extensive fetal and placental tissue growth of normal pregnancy is characterized by a strong local demand for vascular expansion, but so far little is known about factors regulating VEGF in human pregnancy.

The absence of detectable VEGF immunoreactivity in maternal serum in early and term pregnancy, followed by a post-partum rise in serum VEGF concentrations, suggested the presence of a pregnancy associated factor suppressing VEGF immunoreactivity in ELISA. Measurement of the recovery of VEGF added to serum showed the presence of a factor which probably bound VEGF. This activity was also detected in amniotic fluid at much higher concentrations. Gel filtration of [ $^{125}$ I]VEGF added to pregnancy serum and amniotic fluid showed that this binding activity occurred in the high molecular weight fractions.

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In early pregnancy, the VEGF-binding capacity was about 50 times higher in amniotic fluid than in maternal serum. The binding capacity in term amniotic fluid was two-fold that of early amniotic fluid. The origin and source, as well as the exact nature of the binding compound need further studies, but from the above data it can be concluded that the binding activity is strongly associated with gestation and dependent on gestational age. The high concentrations of the binding factor in amniotic fluid suggest that it originates from the amniotic fluid compartment. Interestingly, in gel filtration the complex formed in early amniotic fluid showed two distinct components, whereas term amniotic fluid contained only a single high molecular weight complex corresponding to the later eluting compound in early amniotic fluid.

The identity of the VEGF-binding compound is not yet known, but well known proteins of the corresponding molecular weight can be excluded.  $\alpha 2M$ , which is a major protease inhibitor in serum, has been shown to bind VEGF irreversibly (Soker *et al.*, 1993), and in the immunodiffusion studies some radioactivity was found to be associated with  $\alpha 2M$ , but this could not be detected in amniotic fluid. Assays of  $\alpha 2M$  by IFMA confirmed that it is not detected in amniotic fluid (Bhat and Parthibanam, 1980). Heparin did not affect VEGF binding in amniotic fluid or serum of pregnant women, whereas it has been reported to inhibit its binding by  $\alpha 2M$  (Soker *et al.*, 1993). These results indicate that  $\alpha 2M$  is not responsible for the VEGF binding activity in amniotic fluid. However, on the basis of our immunodiffusion results,  $\alpha 2M$  binds some VEGF in serum of non-pregnant subjects, and it probably contributes to a minor part of the binding activity in maternal serum.

The serum concentrations of two high molecular weight proteins, PZ and PAPP-A have been shown to increase with advancing pregnancy, reaching peak at term, and declining during the post-partum days (von Schoultz, 1974; Lin *et al.*, 1976). PAPP-A is also present in amniotic fluid (Bischof *et al.*, 1982). We therefore studied the possible role of these proteins in VEGF-binding activity. In immunodiffusion neither PZ nor PAPP-A reacted with complexed VEGF. Furthermore, the molecular weight and isoelectric point (pI) of the complex, approximately 440 kDa and pI approximately 8, differ from those of PAPP-A,  $M_r$  820 kDa (Sinosich *et al.*, 1980) and pI 4.4 (Lin *et al.*, 1976) and from the molecular weight of PZ,  $M_r$  326 kDa (von Schoultz, 1974).

We studied whether the soluble form of VEGFR-1 (Kendall and Thomas, 1993; Kendall *et al.*, 1996; Banks *et al.*, 1998) would be responsible for the binding of VEGF by measuring the recovery of PlGF. VEGFR-1 binds PlGF with high-affinity (Park *et al.*, 1994) but no inhibition of PlGF immunoreactivity in ELISA was observed. Therefore it seems unlikely that soluble VEGFR-1 is responsible for the VEGF-binding activity observed in amniotic fluid. Furthermore, the molecular size of the soluble VEGFR-1 is only about 90 kDa (Kendall and Thomas, 1993).

The nature of binding of VEGF to the complex in amniotic fluid was characterized by studying the ability of high salt, low pH, heparin and the chaotropic salt KSCN to release [ $^{125}I$ ]VEGF from the complex. [ $^{125}I$ ]VEGF was not released from the complex either by high concentrations of NaCl or by

heparin, but 4 mol/l KSCN and low pH caused dissociation of the complex. Taken together, these results indicate that VEGF is bound strongly by an acid stable protein. In amniotic fluid, the binding protein is heterogeneous in molecular size. Such a size heterogeneity can be due to dimerization, but the existence of two separate binding proteins cannot be excluded. Interestingly, the dimeric  $\alpha 2M$  molecule forms a tetramer when it reacts with proteases. However, VEGF is not known to exert proteolytic activity and protease- $\alpha 2M$  complexes are covalent.

It has recently been suggested that VEGF-binding protein or proteins occur in maternal serum and that such binding proteins were also present in blood from non-pregnant subjects and the umbilical vein, but were saturated to leave unbound VEGF available for detection by ELISA (Anthony *et al.*, 1994). The results of this study suggest that the VEGF-binding activity of umbilical cord and post-partum maternal samples is low, and that most of the VEGF is unbound.

In conclusion, a putative heterodimeric high molecular weight VEGF-binding protein is present in maternal serum and amniotic fluid. Its concentrations are dependent on gestational age, it disappears after delivery, and it is not clearly detectable in umbilical blood. Because it does not bind PlGF, it does not appear to be the presently known soluble VEGF receptor. Further studies are needed to identify this VEGF-binding compound and to elucidate its potential role in the regulation of vasculogenesis of human pregnancy.

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## Soluble VEGFR-1 secreted by endothelial cells and monocytes is present in human serum and plasma from healthy donors

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### Abstract

It was shown before that the soluble form of VEGFR-1 (sVEGFR-1) is present in serum of pregnant women. The aim of the present study was to investigate the presence of this endogenous vascular endothelial growth factor-A (VEGF-A) antagonist in human serum in more detail. sVEGFR-1 was detected in human serum and plasma from normal healthy male and female donors by ELISA. sVEGFR-1 levels ranged from non-detectable up to 440 pg/ml, with no significant difference between male and female donors. In addition, vein endothelial cells (ECs) from an intact vascular bed, the umbilical cord, were shown to secrete sVEGFR-1. Furthermore, human peripheral blood monocytes, a non-EC type expressing VEGFR-1, were shown to contribute to the sVEGFR-1 detectable in human serum and plasma for the first time. EC- and monocyte-derived sVEGFR-1 proved capable of inhibiting the VEGF-induced proliferation and migration of ECs *in vitro*. Finally, secretion of sVEGFR-1 was increased by the angiogenic factor basic fibroblast growth factor (bFGF) in human ECs and was also enhanced in lipopolysaccharide-activated human monocytes. In human umbilical vein endothelial cells, both the membrane-bound and the sVEGFR-1 seem to be equally regulated on the mRNA as well as the protein level. The presence of an sVEGFR-1 in human serum and plasma of normal male and female donors strongly suggests that it plays an important role as a naturally occurring VEGF antagonist in the regulation and availability of VEGF-mediated biological activities *in vivo*.

**Abbreviations:** CM – conditioned medium; EC – endothelial cell; LPS – lipopolysaccharide; MOI – multiplicity of infection; PMA – phorbol 12-myristate 13-acetate; Sf9 cells – *Spodoptera frugiperda* cells; VEGF – vascular endothelial growth factor

### Introduction

Angiogenesis, the development of new blood vessels from an existing vascular bed, is not only essential for many physiological processes, e.g. ovulation and placenta, but also in pathophysiological situations such as tumor angiogenesis, diabetic retinopathy, rheumatoid arthritis, or psoriasis [1–4]. Several factors have been identified with angiogenic activity including the vascular endothelial growth factor-A (VEGF-A), which is a potent and specific mitogen for endothelial cells (ECs) [5, 6]. The pivotal role of the VEGF-A receptor system in angiogenesis is illustrated by abnormal blood vessel

development and embryonic lethality in gene knockout experiments [7, 8].

Structurally, VEGF-A is a member of the platelet-derived growth factor (PDGF) family together with other related factors including VEGF-B, -C, -D, -E, and placenta growth factor (PlGF) [5, 6]. Two high-affinity tyrosine kinase receptors for VEGF-A have been identified, VEGFR-1 (FLT-1 [9]), and VEGFR-2 (KDR [10]). Consistent with the EC-specific action of VEGF-A, expression of both receptor genes has been found predominantly but not exclusively on ECs [11]. Expression of VEGFR-1 was also found on human monocytes, neutrophils (PMNs), bovine brain pericytes and villous and extravillous trophoblasts [12–14]. Recently, a soluble form of the VEGFR-1 (sVEGFR-1) has been identified in conditioned medium of cultured human umbilical vein ECs (HUVECs) [15]. Soluble forms of several receptor tyrosine kinases have been described so far, e.g. basic fibroblast growth

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factor (bFGF) receptor-1 [16], angiopoietin receptor TIE-1 [17], PDGF receptor- $\alpha$  [18] and epidermal growth factor receptor [19]. Two different mechanisms seem to be responsible for the formation of the soluble receptors. The first mechanism involves limited proteolysis in the extracellular region of the membrane-bound receptor, a process termed 'shedding'. In this process a proteinase cleaves a juxtamembranous peptide bond in the extracellular domain of the membrane receptor leaving the ligand-binding domain intact. At present there is no general amino acid sequence (consensus sequence) known that is recognized by the proposed shedding proteinase. In the case of TIE-1 it was shown that soluble TIE-1 is released through the activation of an endothelial membrane-associated metalloproteinase [20]. Second, soluble receptors can be translated from differentially spliced pre-mRNA molecules lacking the transmembrane domain. The difference between the nucleotide sequences of the membrane-bound and the soluble receptors results from either insertion or deletion of nucleotide sequences, reflecting two types of differential splicing. The soluble receptors differ from the extracellular region of their membrane-bound counterparts by missing extracellular amino acids and by having unique amino acids added at the C-terminus. The sVEGFR-1 is generated by the second mechanism, the alternative splicing of a single gene. sVEGFR-1, already predicted in the original report by Shibuya et al. [21], consists solely of the N-terminal six of the seven extracellular Ig-like domains fused to the unique intron-encoded 31-amino acid residue C-terminal sequence [22]. The sVEGFR-1 is a heparin-binding protein that binds VEGF-A with the same high affinity as the full-length membrane-spanning receptor and inhibits the mitogenic response to VEGF-A in culture by sequestering VEGF-A [15]. Very recently, this sVEGFR-1 was shown to be present in serum of pregnant women but was not detectable in serum from men or non-pregnant women [14].

Here we show that this sVEGFR-1 is also detectable in human serum and plasma samples from normal healthy male and female donors. We demonstrate that besides cultured primary vascular ECs, vein ECs from an intact umbilical cord are also able to secrete sVEGFR-1. In addition, sVEGFR-1 was also detectable in CM of freshly isolated human monocytes. Naturally occurring sVEGFR-1 competed in a dose-dependent manner for binding of VEGF-A with cell-surface VEGF receptors and inhibited the VEGF-A-induced mitogenesis and chemotactic response in ECs. Finally, secretion of sVEGFR-1 is enhanced in activated HUVECs and monocytes. We also provide the first evidence that in HUVECs both the membrane-bound and the sVEGFR-1 are equally regulated on the mRNA as well as on the protein level. These results suggest that sVEGFR-1 might have an important function in the regulation of VEGF-A-mediated biological activities *in vivo*.

## Materials and methods

### Cell culture and reagents

Primary cultures of ECs from HUVECs and human umbilical artery ECs (HUAECs) were isolated from fresh umbilical cords by mild digestion with dispase II (0.6 units/ml; Boehringer Mannheim, Germany). Human dermal microvascular ECs (HDMVECs), human kidney venous ECs (HKVECs), and human microvascular ECs from normal (HKMVECs) and malignant (HRMVECs) kidney tissue were isolated using standard isolation procedures (B. Barleon, unpublished data). All ECs were grown in endothelial growth medium (EGM) (PromoCell, Heidelberg, Germany) supplemented with 3% fetal calf serum (FCS). For stimulation experiments, cells were grown in endothelial basal medium (EBM/1% FCS) for 24 h, and then factors were added. Human primary cultures of dermal fibroblasts were isolated using standard procedures. Primary cultures of human keratinocytes and osteoblasts were a friendly gift from the Department of Plastic and Hand Surgery, University of Freiburg. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of normal donors (courtesy of the Transfusion Center, University Hospital, Freiburg) as described [23]. VEGF<sub>165</sub> was expressed using baculovirus system in *Spodoptera frugiperda* (Sf9) insect cells, and was iodinated to a specific activity of 100,000 cpm/ng [24, 25]. The polyclonal anti-VEGFR-1 antiserum was raised against recombinant sVEGFR-1 (rsVEGFR-1(5)) in rabbits. The monoclonal anti-VEGFR-1 antibodies were directed against rsVEGFR-1(7) in rat (7A6) or rsVEGFR-1(5) in mouse (11G2, 4C8-10) as described [26]. The monoclonal VEGFR-2 antibody 3G2 was directed against rsVEGFR-2(7), while the monoclonal TIE-1 (7E3) and TIE-2 (#16) antibodies were directed against the full extracytoplasmatic domain of recombinant sTIE-1 and sTIE-2 proteins in mouse (P. Reusch, unpublished data). Phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co. (St Louis, Missouri), bFGF was from Saxon Biochemical (Hannover, Germany), and bacterial lipopolysaccharide (LPS D1, *Escherichia coli* 055:B5) was from Difco Labs (Detroit, Michigan). N-glycosidase F was purchased from Boehringer Mannheim (Germany).

### Cloning of sVEGFR-1 by RT-PCR from total RNA of HUVECs

Five microgram of HUVEC total RNA was reversely transcribed with the cDNA synthesis kit (Pharmacia, Germany) and subjected to PCR. The following sense and antisense primers were used for gene amplification: sense primer hsft-1: 5'-GGAATTCGCGCTCAC-CATGGTCAGCTACTGG-3', containing an *EcoRI* site; antisense primer hsft-2: 5'-ATGGATCCTTTAA-TGTTTTACATTACTTTGTGTGG-3', containing a *BamHI* site. PCR product corresponds to nucleotide

240–2317 of the published human sVEGFR-1 cDNA sequence [22]. PCR was performed using the following thermal profile for 40 cycles: 94 °C for 45 s, 53 °C for 2 min, and 75 °C for 3 min. The purified PCR product was subcloned into the baculovirus transfer vector pVL1392 as a *EcoRI/BamHI* fragment. Plasmids containing the cDNA were isolated from transformed bacteria and used for transfection into Sf9 cells along with wild-type baculovirus DNA. Recombinant baculoviruses were obtained using the Baculogold™ transfection kit following standard protocols (Pharmingen, USA). For protein expression, Sf9 cells at a density of  $2 \times 10^6$  cells/ml were infected with a multiplicity of infection (MOI) of 10. RsVEGFR-1 was used as positive control in the Western analysis and for the calibration curve in the ELISA [27].

*Preparation of cell supernatants and partial purification of sVEGFR-1 by heparin-affinity chromatography*

For collection of conditioned medium (CM) from HUVECs, cells were grown to near confluence, medium was changed against EBM/1% FCS, and harvested 24–30 h later. For CM from PBMCs, freshly isolated monocytes were plated in RPMI-1640/1% FCS (Gibco, Germany) and allowed to condition their medium for up to 72 h. CM was harvested, spun down for 20 min at 4000 rpm and filtered. For purification of sVEGFR-1, CM (80–160 ml) was adjusted to pH 6.4 and applied to a 1 ml heparin–Sephacrose column (Pharmacia, Germany). The column was washed once with 10 ml of 0.4 M NaCl/20 mM Tris, pH 7.4 and bound proteins were eluted by a step-gradient using 0.5–2.0 M NaCl/20 mM Tris, pH 7.4 as described [26]. For collection of CM from intact human umbilical cords, the veins of fresh umbilical cords were first rinsed with PBS to remove residual blood cells, perfused with EBM/1% FCS, sealed and incubated for up to 24 h. The CM was harvested, cleared by centrifugation and then assayed for sVEGFR-1 activity by ELISA. For collection of CM from the different primary human-ECs and non-ECs, normal growth medium was harvested after 48–72 h, cleared by centrifugation and then assayed for sVEGFR-1 activity by Western analysis and ELISA.

*Immunoprecipitation and immunoblotting*

For immunoprecipitation, samples were incubated with 2 µg anti-VEGFR-1 antibodies together with 30 µl of anti-rat or anti-mouse IgG–agarose beads (Sigma, Germany) on a rotating dish for 3 h at 4 °C. Agarose beads were spun down by centrifugation, washed four times in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5), resuspended in 2× SDS sample buffer and resolved by SDS/PAGE (10%). The gels were electroblotted onto a PVDF membrane, blots were blocked for 30 min with 5% milk powder in TBST and incubated for 1 h in the same buffer with 1 µg/ml of anti-VEGFR-1 antibodies. The blots were washed twice

in water and TBST and incubated for 45 min in TBST containing peroxidase-conjugated secondary antibodies. The blots were washed as described before and peroxidase coupled antibodies were visualized using the ECL chemiluminescent Western blotting detection system (Amersham, Germany).

*Binding assay*

Competition binding experiments on HUVECs were done as described [28]. Briefly, HUVECs (passage 4) were seeded in growth medium at  $5 \times 10^4$  cells/well in 24-well cluster plates. After 72 h the cells were washed extensively with binding buffer (DMEM, 25 mM HEPES, 1 mg/ml BSA, pH 7.4) and incubated for 3 h at 4 °C with binding buffer containing 1 ng/ml of  $^{125}\text{I}$ -VEGF<sub>165</sub>. Increasing amounts of VEGF<sub>165</sub> or sVEGFR-1, partially purified from HUVEC-CM, were used for competition. The cells were washed three times with binding buffer, solubilized with 250 µl 0.3 M NaOH, 1% SDS per well, and radioactivity present in the lysates was quantified.

*Assay for mitogenic and migratory activity*

Thymidine incorporation assays were essentially performed as described [26]. HUVECs (passage 3) were seeded at a density of  $7 \times 10^4$  cells/well in 48-well cluster plates and cultured over night in EGM/5% FCS. The next day medium was changed against basal medium (EBM/1% FCS). Twenty-four hours later the cells were stimulated with 3 ng/ml VEGF<sub>165</sub> (determined to be sufficient for a maximal response by dose-response experiments) together with increasing amounts of sVEGFR-1 or rsVEGFR-1. As a control HUVECs were also stimulated with 10 ng/ml bFGF together with the highest amount of rsVEGFR-1 used for VEGF<sub>165</sub>. Eighteen hours later 0.5 µCi [ $^3\text{H}$ ] thymidine (56.7 Ci/mmol; NEN) was added. Cells were kept at 37 °C for an additional 6 h. Cell monolayers were fixed with methanol, washed with 5% trichloroacetic acid, solubilized in 0.3 M NaOH and counted by liquid scintillation. Cell migration assays were performed using 8.0 µm pore size Transwells (Costar Corp., Germany) as described [29]. The inserts were precoated with 1.5% gelatine in water for 30 min before placement of the cells into the insert. Migration was induced by VEGF<sub>165</sub> (5 ng/ml) and measured after 4 h of incubation at 37 °C. Non-specific migration was determined by adding the carrier solution without stimuli.

*VEGFR-1-specific ELISA*

The sVEGFR-1-specific ELISA was essentially performed as described previously [27]. The mouse sVEGFR-1 mab 11G2 was used as capture antibody at a concentration of 2 µg/ml. Serum and plasma samples were used undiluted, the CM of the primary cells were 1:5 diluted in the same medium. For detection

of the membrane-bound receptor, HUVECs were lysed in lysis buffer containing a set of protease inhibitors (Pefablock, Leupeptin, NaF, Na vanadate). Protein concentration in lysates was determined by BCA assay (Pierce). The lysates were diluted 1:2 in PBS. Hundred microliter of samples per well were used in the assay. A calibration curve was setup in parallel using purified recombinant sVEGFR-1. The statistical analysis of the data were performed by an unpaired *t*-test analysis using the GraphPad Prism 3.0 software.

#### Northern analysis

Total RNA was prepared with the RNeasy™ total RNA kit (Qiagen), separated in formaldehyde-containing agarose gels, transferred to nitrocellulose membranes, and hybridized to <sup>32</sup>P-labeled cDNA fragments using standard protocols. The specific cDNA probe for VEGFR-1 was a human 1.05 kb cDNA fragment (nucleotides 172–1231) [28]. To control for sample loading and RNA integrity, the filter was rehybridized with a 1.2 kb human GAPDH cDNA probe.

#### Blood sampling

A group of 10 healthy volunteers (5 female, 5 male) was recruited for the study after informed consent. For each time point, venous blood (20 ml) was collected into Sarstedt tubes without anticoagulant and for plasma collection using EDTA and citrate as anticoagulant. Within 30 min of venepuncture, samples were centrifuged at 2000 × *g* for 15 min, the serum and plasma removed, aliquoted and stored at –80 °C until analysis. For each individual, eight samples were collected during 1 week to define intraindividual variations of receptor levels. For depletion of serum, anti-mouse IgG agarose (Sigma, Germany) was preincubated with 3 µg monoclonal antibody against human receptors TIE-2, VEGFR-2/KDR, VEGFR-1/FLT-1 or TIE-1 followed by incubation with human serum (2 h at room temperature). After centrifugation, the depleted serum (supernatant) was analyzed for sVEGFR-1 concentration by sandwich ELISA.

## Results

#### Detection of sVEGFR-1 activity in human serum and plasma

We have improved our sVEGFR-1-specific ELISA [27] for a direct measurement of sVEGFR-1 activity in

human body fluids. The limit of quantitation for this assay is 100 pg/ml. sVEGFR-1 below this limit is still detectable, but quantitation becomes rather imprecise (data not shown). Serum, EDTA- and citrate-plasma samples from 10 healthy volunteers (five males, five females) were used for assay characterization. From each individual donor, eight independent blood samples of each kind were taken within 1 week and tested by ELISA. The serum and plasma samples showed a broad range of sVEGFR-1 content, from not detectable up to about 440 pg/ml (Table 1). However, sVEGFR-1 activity was detectable in independent samples of all volunteers (Figures 1A, B). The mean amount of sVEGFR-1 was 166 ± 39 pg/ml in serum, 125 ± 69 pg/ml in EDTA-plasma, and 109 ± 45 pg/ml in citrate-plasma, respectively. There was no significant difference in the mean amount of sVEGFR-1 in serum (*P* = 0.2888) and citrate-plasma (*P* = 0.2094) samples between female and male donors. In serum, the mean was 156 ± 48 pg/ml for women, and 176 ± 30 pg/ml for men. In citrate-plasma, the mean was 99 ± 37 pg/ml for women and 118 ± 54 pg/ml for men. In contrast, the unpaired *t*-test revealed a significant difference in the mean amount of sVEGFR-1 (*P* = 0.0047) between males and females in the EDTA-plasma samples. In EDTA-plasma the mean was 97 ± 76 pg/ml for women and 154 ± 76 pg/ml for men. The specificity of the observed signal was tested in some selected serum samples from other volunteers by depletion of sVEGFR-1 using several specific and non-specific monoclonal antibodies for immunoprecipitations. The specific anti-sVEGFR-1 antibodies, 4C8-10 and 11G2, caused a complete disappearance of the sVEGFR-1 signal in the ELISA, whereas mab's directed against the extracellular domains of VEGFR-2 (3G2), TIE-1 (7E3) and TIE-2 (#16), respectively, had no effect (Figure 1C). These results strongly indicate that sVEGFR-1 occurs in serum and plasma of normal healthy male and female donors *in vivo*.

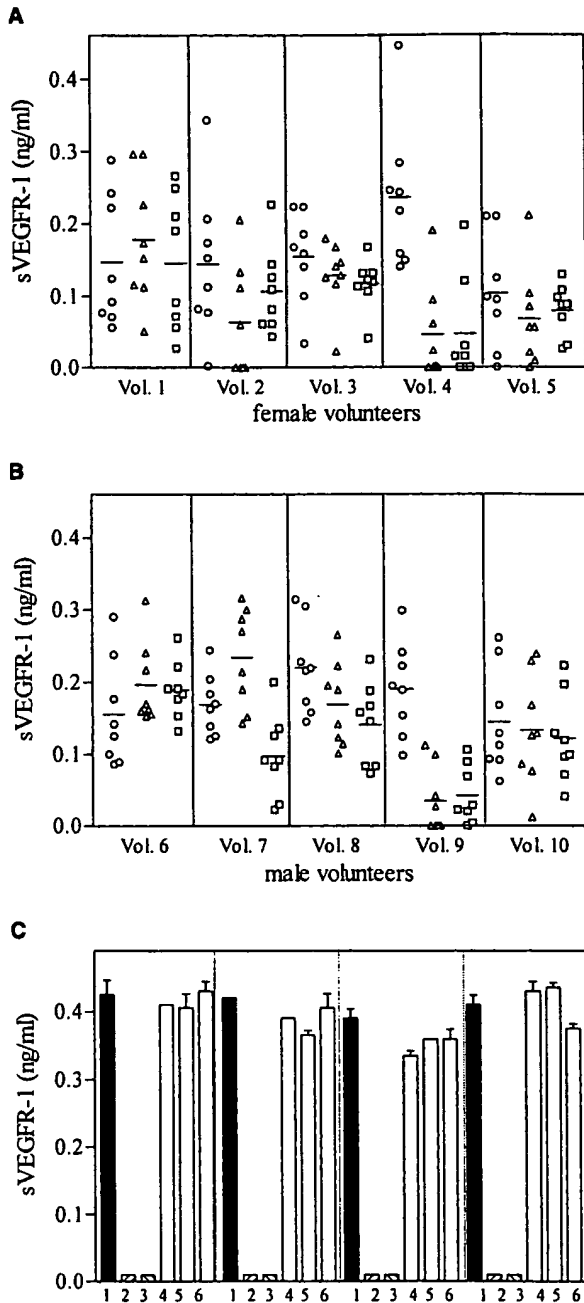
#### Human monocytes secrete a functionally active sVEGFR-1

Subsequently, the question which other cell type(s) express and secrete sVEGFR-1 was addressed. We therefore tested CM derived from peripheral blood mononuclear cells (PBMC), a non-EC type expressing VEGFR-1 [12]. Freshly isolated human monocytes were cultured in RPMI-1640/1% FCS and allowed to condition the medium for 72 h. sVEGFR-1 from monocyte-derived CM was partially purified using a heparin-

Table 1. sVEGFR-1 in serum, EDTA-plasma and citrate-plasma of healthy volunteers.

	Material (ng/ml)	Serum sVEGFR-1	EDTA-plasma sVEGFR-1	Citrate-plasma sVEGFR-1
Female volunteers ( <i>n</i> = 5)	Range	0.0–0.446	0.0–0.296	0.0–0.26
	Mean ± SD	0.156 ± 0.048	0.097 ± 0.076	0.099 ± 0.037
Male volunteers ( <i>n</i> = 5)	Range	0.062–0.310	0.0–0.310	0.0–0.260
	Mean ± SD	0.176 ± 0.030	0.154 ± 0.076	0.118 ± 0.054

sVEGFR-1 levels were defined by sandwich ELISA for female (*n* = 5) and male (*n* = 5) individuals.



**Figure 1.** Detection of sVEGFR-1 in human serum and plasma. Serum (O), EDTA-plasma (Δ) and citrate-plasma (□) from 10 healthy volunteers (5 female (A), 5 male (B)) were assayed for the presence of sVEGFR-1 activity by ELISA. 100  $\mu$ l undiluted samples per well ( $n = 2$ ) were used in the assay. The mean of eight independent samples are indicated by a string. (C) To check for specificity, serum was depleted by immunoprecipitations with several specific and non-specific monoclonal antibodies prior to ELISA. Hundred microliter per well of the supernatant were used in the assay. (1): serum sample; (2) and (3): sVEGFR-1-specific mab 4C8-10 and 11G2; (4) to (6): sKDR-specific mab 3G2; sTIE-1-specific mab 7E3; sTIE-2-specific mab #16. Values are the means ( $\pm$  SD) of triplicate determinations ( $n = 3$ ).

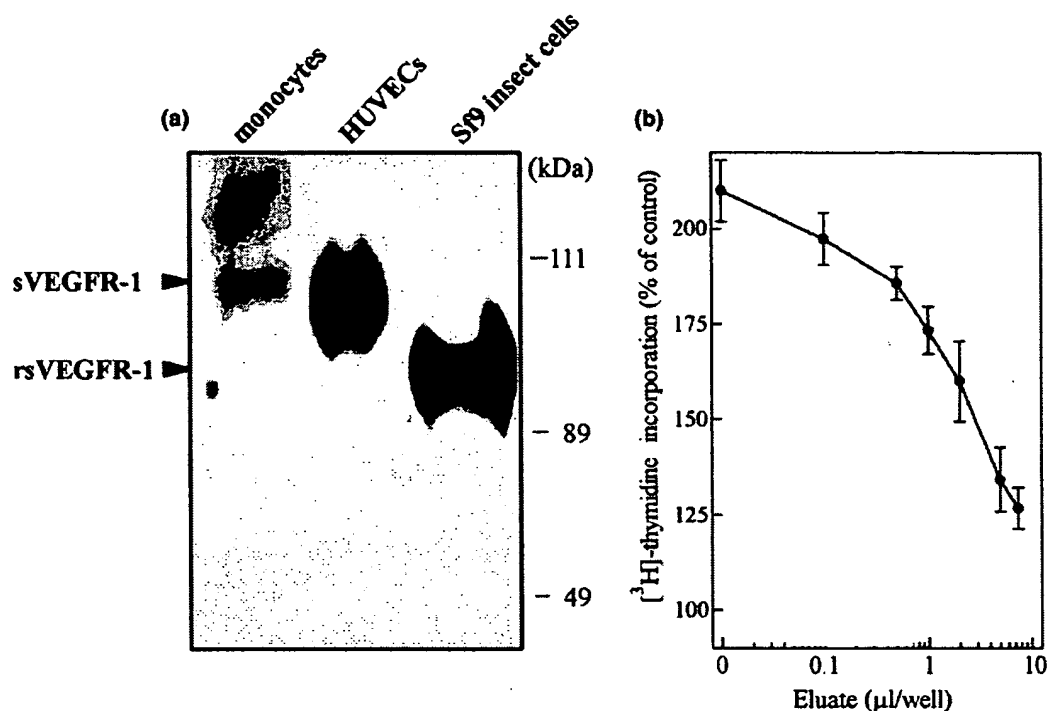
Sephacrose affinity matrix. Positive fractions were determined by Western blotting using the rat mab 7A6 against sVEGFR-1 [26]. As shown in Figure 2A, freshly isolated human monocytes secrete a sVEGFR-1-specific protein with an apparent molecular weight of about 108 kDa, which is identical to the size of sVEGFR-1 secreted by HUVECs [22]. Due to the weaker glycosylation found in insect cells, Sf9-expressed rsVEGFR-1 had an apparent molecular weight of about 96 kDa [15]. Deglycosylation of native and rsVEGFR-1 with N-glycosidase F [26] decreased the molecular size of both proteins to about 76 kDa (data not shown). This size is consistent with the molecular weight calculated from the deduced amino acid sequence [22]. The amount of sVEGFR-1 secreted by human monocytes was in the range of about 1–5 ng/ml as determined by ELISA (data not shown). To investigate the activity of monocyte-derived sVEGFR-1, we performed DNA synthesis experiments using increasing volumina of an sVEGFR-1 positive elution fraction to inhibit the VEGF-A-induced mitogenic response in HUVECs. Figure 2B shows that the monocyte-derived sVEGFR-1 is biologically active, inhibiting the VEGF-A-induced thymidine-uptake into HUVECs in a dose-dependent manner.

#### Secretion of sVEGFR-1 by several human ECs

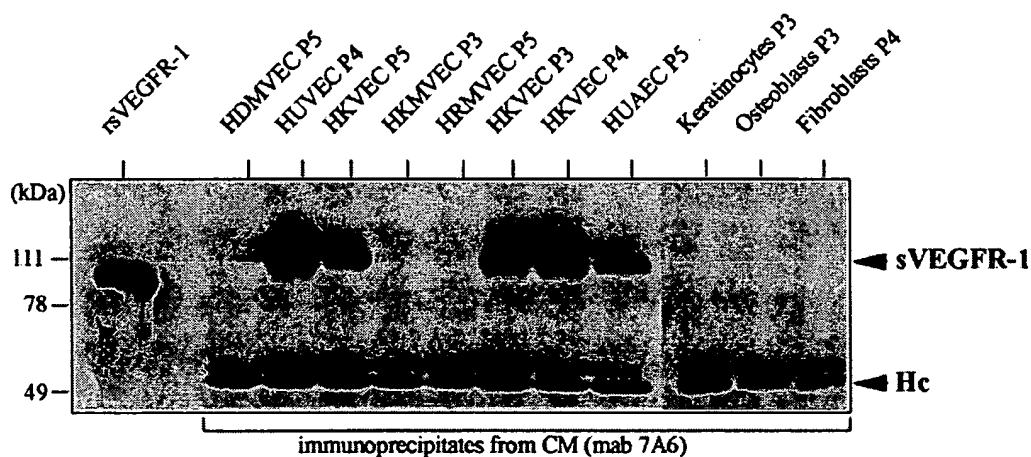
We also tested CM derived from several other primary human-ECs and non-ECs for sVEGFR-1 by Western analysis. CM was harvested from HUAECs and HUVECs, HDMVECs, HKVECs, HKMVECs and HRMVECs. From non-ECs, CM was harvested from keratinocytes, osteoblasts and dermal fibroblasts. All CM (48–72 h) was harvested at the routine medium change and frozen at  $-20^{\circ}\text{C}$  until use. Immunoprecipitation (2–10 ml of CM) was performed with the rat mab 7A6 (2  $\mu\text{g}/\text{ml}$ ), and for the subsequent Western analysis the mouse mab 11G2 (1  $\mu\text{g}/\text{ml}$ ) was used as detection antibody. Purified rsVEGFR-1 was used as positive control. As shown in Figure 3, all CM from ECs were positive for sVEGFR-1. As expected, the CM of the non-ECs were negative for sVEGFR-1 protein. Due to the randomly harvested CMs, the range of sVEGFR-1 content in the different CM was very broad, between 1.6 and 56 ng/ml as determined by ELISA (data not shown). However, these results indicate that most likely all cells expressing VEGFR-1 are capable of secreting sVEGFR-1.

#### Secretion of sVEGFR-1 by ECs of veins in intact human umbilical cords

We then addressed the question whether sVEGFR-1, detected in human serum and plasma, is secreted solely by monocytes or also by ECs *in vivo*. For this purpose we investigated the secretion of sVEGFR-1 by ECs in an *in vitro* setting as close as possible to the *in vivo* situation. The veins of fresh intact human umbilical



**Figure 2.** Detection of sVEGFR-1 in monocyte-conditioned medium. Freshly isolated human monocytes were cultured in RPMI-1640/1% FCS and allowed to condition the medium for 72 h. CM was run through a heparin-Sepharose column, and bound proteins were eluted with a NaCl gradient. (A) Western analysis of the 1 M NaCl elution fraction with the sVEGFR-1-specific mab 7A6 (lane 1). HUVEC-derived sVEGFR-1 (lane 2) and rsVEGFR-1 (lane 3) were used as positive controls. (B) Effect of an sVEGFR-1-positive fraction on the VEGF-A-induced DNA synthesis of HUVECs. DNA synthesis was stimulated by the addition of 3 ng/ml VEGF<sub>165</sub> and the incorporation of [<sup>3</sup>H]-thymidine was determined. Values are the means (±SD) of triplicate determinations at each eluate volume and expressed as percentage of control.



**Figure 3.** Secretion of sVEGFR-1 by several primary human ECs. Various primary human ECs and non-ECs were cultured in the appropriate growth medium. Medium was harvested at the routinely medium change and assayed for sVEGFR-1 protein. The CM of the non-ECs was used as negative control. Western analysis of immunoprecipitates from CM (2–10 ml) of several human ECs and non-ECs. Human rsVEGFR-1 (lane 1) was used as positive control. Primary cells: HDMVEC – human dermal microvascular ECs; HUVEC – human umbilical vein ECs; HKVEC – human kidney venous ECs; HKMVEC – human kidney microvascular ECs; HRMVEC – human renal microvascular ECs; HUAEC – human umbilical artery ECs.

cords ( $n = 20$ ) were first rinsed with PBS to remove residual blood cells, perfused with EBM/1% FCS, sealed and incubated for up to 24 h. The CM was then assayed for sVEGFR-1 activity by ELISA. Uncondi-

tioned medium was used as negative control. HUVECs from an intact vascular bed are able to secrete sVEGFR-1 into the medium, ranging from about 2–22 ng/ml (mean:  $11.38 \pm 6.49$  ng/ml) (data not shown). To verify

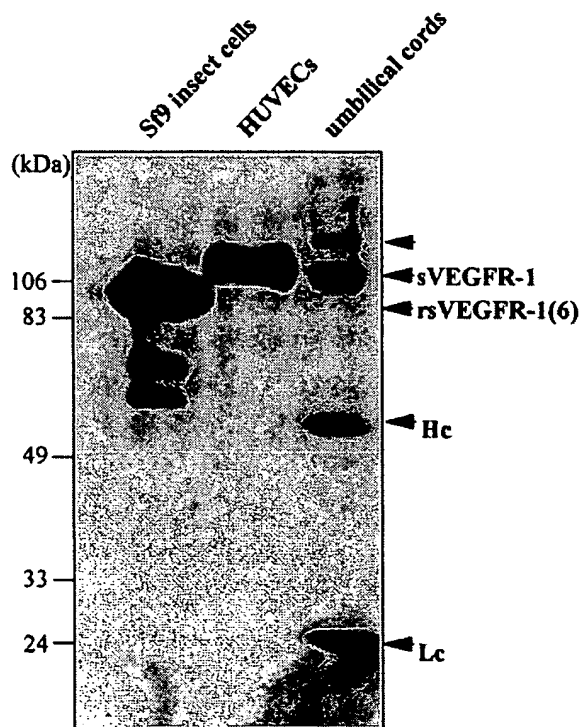
that the sVEGFR-1 specific protein is identical with those secreted by monocytes and HUVECs, we performed an immunoprecipitation followed by Western blotting. A sVEGFR-1-specific protein with an apparent molecular weight of about 108 kDa was detectable in CM-derived from vein ECs of intact human umbilical cords (Figure 4). The size of 108 kDa is identical to those of sVEGFR-1 secreted by monocytes and that reported by Kendall et al. [15]. Because VEGFR-1 and sVEGFR-1 are not expressed in smooth muscle cells and fibroblasts, at least on the mRNA level [28], these results strongly indicate that ECs from an intact vascular bed are capable of secreting sVEGFR-1 *in vivo*.

#### Characterization of naturally occurring sVEGFR-1

In order to investigate VEGF-A binding properties and biological activity of naturally occurring sVEGFR-1 in more detail, we purified sVEGFR-1 from HUVEC-CM as described [26]. The concentration of the partially purified sVEGFR-1 was estimated by Western blotting using purified rsVEGFR-1 as a reference. In a first approach we performed a cell-based competition binding assay with primary human ECs as target. Increasing

amounts of rVEGF<sub>165</sub> and sVEGFR-1 were used for competition. We could show that endogenous sVEGFR-1 partially purified from HUVEC-CM competed with the membrane-bound VEGF receptors for binding of <sup>125</sup>I-VEGF<sub>165</sub> with a similar affinity (Figure 5A). As expected from this result, HUVEC-derived sVEGFR-1 already inhibited the [<sup>3</sup>H]-thymidine incorporation in HUVECs stimulated by 3 ng/ml VEGF<sub>165</sub> by a 4- to 6-fold molar excess of sVEGFR-1 over VEGF<sub>165</sub> (Figure 5B). We also tested the VEGF-A-induced cell migration using the Transwell system (Costar Corp., Germany). Migration was induced by 5 ng/ml VEGF<sub>165</sub>. For inhibition, VEGF<sub>165</sub> was preincubated either with rsVEGFR-1 or HUVEC-derived sVEGFR-1 (each 1 nM) for 30 min at 37 °C and then added to the lower chamber of the well. In this type of assay we needed a 8- to 10-fold molar excess of sVEGFR-1 over VEGF<sub>165</sub> to get a reduction in cell migration of approximately 50% (Figure 5C). However, these results show that naturally occurring sVEGFR-1 can act as an efficient antagonist of VEGF-A-induced biological activities *in vitro*.

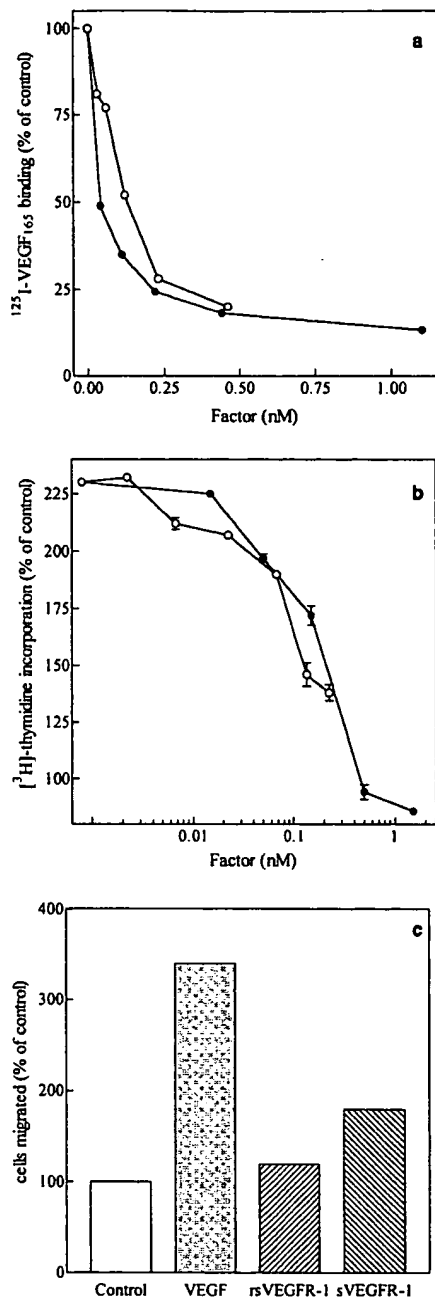
#### Enhanced secretion of sVEGFR-1 by activated HUVECs and monocytes



**Figure 4.** Detection of sVEGFR-1 in CM from veins in intact human umbilical cords. The veins of intact umbilical cords ( $n = 20$ ) were incubated with EBM/1% FCS for up to 24 h. CM was harvested, pooled and assayed for the presence of sVEGFR-1. Western blotting of immunoprecipitates with the sVEGFR-1-specific mab 7A6 (lane 3). The light (Lc) and heavy (Hc) antibody chains in lane 3 are indicated by arrows. The larger band of about 125 kDa is unknown. HUVEC-derived sVEGFR-1 (lane 2) and rsVEGFR-1 (lane 1) were used as positive controls.

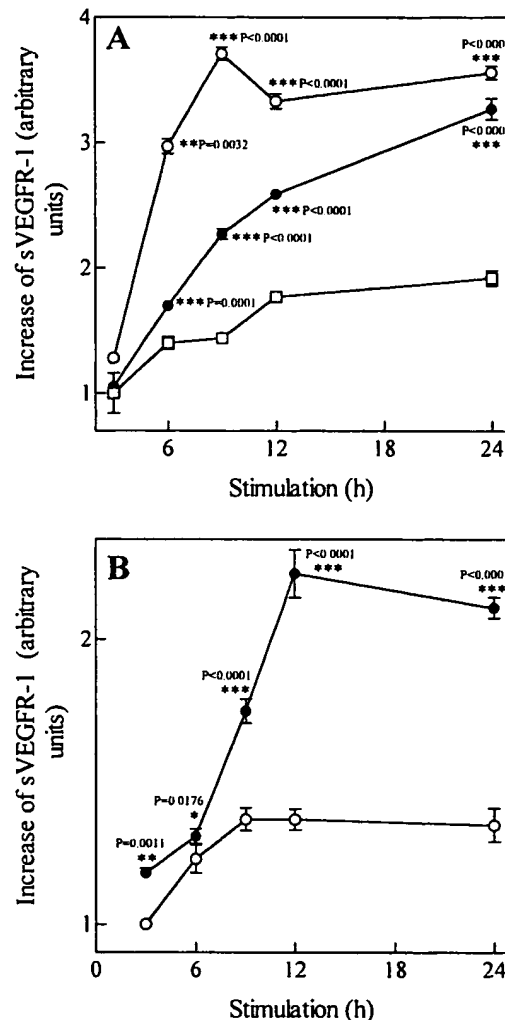
So far, little is known about the physiological stimuli responsible for the generation of soluble forms of growth factor receptors. Therefore, in subsequent experiments we addressed the question whether factors involved in EC proliferation and migration are able to influence the secretion of sVEGFR-1. Recently we have shown that VEGFR-1 and sVEGFR-1 are up-regulated on the mRNA and protein levels in HUVECs by VEGF itself [27]. The VEGFR-1 mRNA level is also up-regulated by the angiogenic factor bFGF and the tumor-promotor PMA. In both cases, the 2.7 kb mRNA transcript, encoding the soluble VEGFR-1, was significantly enhanced [30]. To examine whether the increase of the 2.7 kb mRNA is accompanied by an enhanced secretion of sVEGFR-1 in HUVECs, the cells were cultured in basal medium for 24 h and then stimulated either with bFGF (10 ng/ml) or PMA (25 ng/ml). The CM were harvested, cleared by centrifugation and measured for sVEGFR-1 activity by ELISA. CM of unstimulated cells was used as a control. Stimulation of HUVECs with bFGF or PMA led to an enhanced secretion of sVEGFR-1 into the medium in a time-dependent manner. The bFGF-induced sVEGFR-1 secretion showed a continuous increase over the time, whereas the tumor-promotor PMA reached a maximum at about 9 h. Both factors induced an about 2-fold increase of sVEGFR-1 secretion as compared to the control over a 24 h period (Figure 6A). In unstimulated HUVECs, the actual amount of secreted sVEGFR-1 ranged from 0.8 to 8.0 ng/ml in independent experiments.

It is known that macrophages and their precursor monocytes play a pivotal role in wound repair and immune responses, processes in which VEGF-A is also

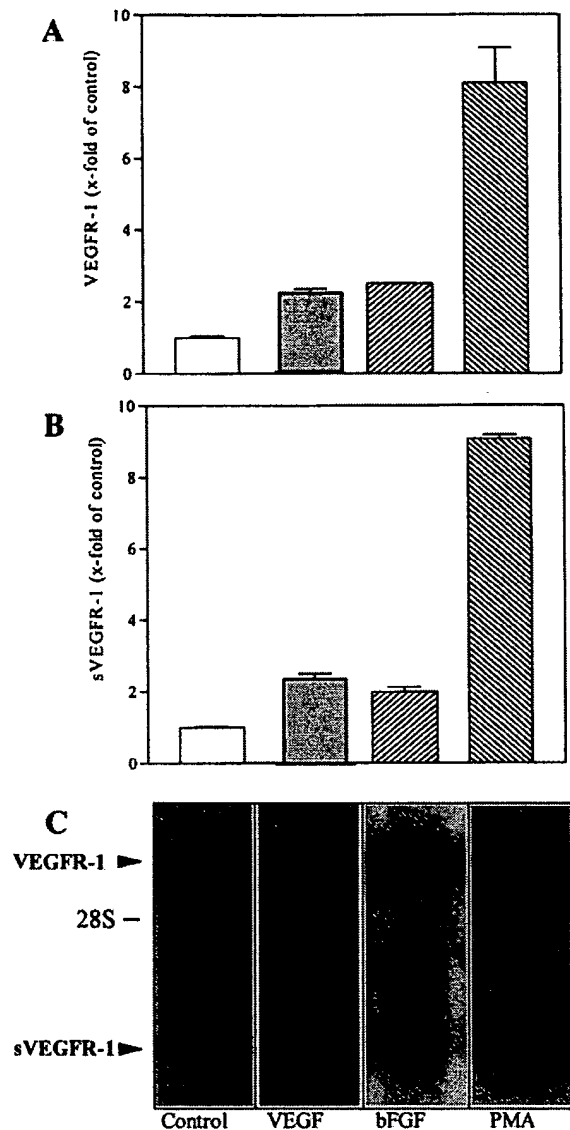


**Figure 5.** Characterization of naturally occurring sVEGFR-1. (A) Competition of VEGF-A binding to cell surface VEGF receptors by HUVEC-derived sVEGFR-1 (O) and VEGF<sub>165</sub> (●). Binding experiments were performed on HUVECs. Increasing amounts of VEGF<sub>165</sub> and HUVEC-derived sVEGFR-1 were used for competition. Total binding was set to 100%. (B) Influence of increasing amounts of partially purified HUVEC-derived sVEGFR-1 (O) and rsVEGFR-1 (●) on the [ $^3\text{H}$ ]-thymidine incorporation, stimulated by 3 ng/ml VEGF<sub>165</sub> on HUVECs. Values are the means ( $\pm$  SD) of triplicate determinations at each sVEGFR-1 concentration and expressed as percentage of control. (C) Effect of partially purified HUVEC-derived sVEGFR-1 and rsVEGFR-1 on the migration of HUVECs, stimulated by 5 ng/ml VEGF<sub>165</sub>. Control was set to 100%. Control (bar 1); 5 ng/ml VEGF<sub>165</sub> (bar 2); 5 ng/ml VEGF<sub>165</sub> + 100 ng/ml rsVEGFR-1 (bar 3); 5 ng/ml VEGF<sub>165</sub> + app. 100 ng/ml sVEGFR-1 (bar 4) ( $n = 2$ ).

involved. We have shown before that activation of human monocytes by LPS, a prototypic monocyte activator [31], causes a significant increase of the VEGFR-1 and sVEGFR-1 mRNA levels [12]. We therefore investigated whether activated monocytes also show an enhanced secretion of sVEGFR-1 protein. Freshly isolated human monocytes, stimulated with 100 ng/ml LPS, showed an about 2-fold increase of secreted sVEGFR-1 (Figure 6B). In unstimulated monocytes, the actual amount of secreted sVEGFR-1



**Figure 6.** Increase of sVEGFR-1 secretion by activated HUVECs and monocytes. (A) HUVEC cultures were serum-starved (1% FCS) overnight and stimulated with bFGF (10 ng/ml) or PMA (25 ng/ml) for the indicated time periods. Control (□); bFGF (●); PMA (○). (B) Freshly isolated monocytes were cultured in RPMI-1640/1% FCS and stimulated with LPS (100 ng/ml) for the indicated time periods. No LPS (□); with LPS (●). CM was harvested and sVEGFR-1 activity was measured by ELISA (100  $\mu\text{l}$ /well). Medium of unstimulated cells was used as control. Control value at timepoint 3 h were set to 1 (HUVEC: 1.2 ng/ml; monocytes: 0.9 ng/ml). Values are the means ( $\pm$ SD) of triplicate determinations for each time point. The statistical analysis of the data were performed by an unpaired *t*-test analysis using the GraphPad Prism 3.0 software.



**Figure 7.** Upregulation of VEGFR-1 and sVEGFR-1 in activated HUVECs. HUVEC cultures were serum-starved (1% FCS) overnight and stimulated either with VEGF<sub>165</sub> (5 ng/ml), bFGF (10 ng/ml) or PMA (25 ng/ml). After 8 h, the CM was harvested, total lysate and total RNA of the cells was prepared. The lysate (A) and CM (B) were assayed for VEGFR-1 and sVEGFR-1 by ELISA. The control value at time point 8 h was set to 1. Values are the means of duplicate determinations for each time point and expressed as fold of control. (C) Northern analysis for expression of VEGFR-1 and sVEGFR-1 in stimulated HUVECs (VEGFR-1: 7.5–8.0 kb; sVEGFR-1: 2.6 kb).

ranged from 0.5 to 2.2 ng/ml in independent experiments. This result strongly indicates that activated human monocytes are capable of secreting sVEGFR-1 *in vivo*.

#### Upregulation of VEGFR-1 and sVEGFR-1 in HUVECs

We then addressed the question how the expression of the full-length VEGFR-1 versus the soluble splice

variant sVEGFR-1 is regulated. Therefore, HUVECs were cultured in basal medium for 24 h and then stimulated with either VEGF<sub>165</sub> (5 ng/ml), bFGF (10 ng/ml) or PMA (25 ng/ml). After 8 h the CM was harvested and the cells were washed twice with PBS and total protein was prepared. The lysate was assayed for the membrane-bound VEGFR-1 protein, the CM for the sVEGFR-1 protein by ELISA. As shown in Figure 7A, B, VEGF<sub>165</sub>, bFGF and PMA induced a similar increase of VEGFR-1 (A) and sVEGFR-1 (B), respectively. VEGF<sub>165</sub> and bFGF induced an about 2-fold increase of VEGFR-1 ( $69.4 \pm 1.2$ ,  $79.5 \pm 1.4$  ng/ $10^6$  cells) and sVEGFR-1 ( $39.1 \pm 1.9$ ,  $32.36 \pm 1.4$  ng/ $10^6$  cells), whereas the tumor-promotor PMA caused a tremendous increase of about 8 to 9-fold ( $258.6 \pm 31.8$ ,  $148.0 \pm 3.0$  ng/ $10^6$  cells). A similar regulation of the full-length VEGFR-1 transcript as well as the 2.7 kb transcript encoding the soluble form we observed on the mRNA level (Figure 7C). These results indicate that both forms of VEGFR-1 are similarly regulated on the mRNA as well as on the protein level.

#### Discussion

In the present study we show that sVEGFR-1 activity is detectable in human serum and plasma from normal healthy male and female donors. Although the range of sVEGFR-1 content in independent samples from the same donor was very broad and even some samples were below the detection limit of the ELISA, all volunteers were found to be positive for sVEGFR-1 in serum and plasma. Clark et al. [14] found sVEGFR-1 to be present in serum from pregnant women but failed to detect sVEGFR-1 in serum from non-pregnant women. These results might be explained by the different sensitivity of the assays used for detection. Our results are based on a very sensitive sVEGFR-1-specific ELISA (detection limit in serum and plasma about 100 pg/ml), whereas Clark et al. [14] analyzed sVEGFR-1 expression by <sup>125</sup>I-VEGF cross-linking studies with heparin-enriched protein fractions from serum of non-pregnant and pregnant women. However, high levels of pregnancy-associated sVEGFR-1 were also shown by others [32, 33]. In addition, the expression of a novel variant of a soluble VEGFR-1 was also found in plasma from pregnant women and in amniotic fluid [34]. Because VEGF-A has been implicated as a key angiogenic factor during pregnancy, the authors speculate that sVEGFR-1 might regulate this activity by sequestering the high amounts of VEGF-A available during this stage in order to protect the mother from inappropriate VEGF-A-induced angiogenic responses [33, 34]. The presence of sVEGFR-1 in serum and plasma of healthy male and female donors strongly indicates that this sVEGFR-1 not only plays a role in pregnancy but might have a more general function in the 'fine' regulation of VEGF-A mediated activities *in vivo*.

This assumption is supported by our finding that primary ECs from different vascular beds, e.g. umbilical



cords, skin, kidney, were capable of secreting sVEGFR-1. In addition, He et al. [33] found by RT-PCR, using specific primers, that sVEGFR-1 transcripts are detectable in adult kidney, lung, uterus and liver. The authors speculated that sVEGFR-1 may have a role in maintaining ECs in a quiescent state in the adult. Furthermore, we were able to demonstrate that peripheral blood monocytes, a non-endothelial but VEGFR-1-expressing cell type, secrete a functionally active sVEGFR-1. These results suggest that vascular ECs, monocytes/macrophages and most likely neutrophils (PMNs), another blood cell type expressing VEGFR-1 [12], contribute to the sVEGFR-1 activity found in human serum and plasma.

An interesting result was the finding that all macrovascular-derived ECs, e.g. HUVECs, HUAECs, and HKVEC, showed a higher expression of sVEGFR-1 than the microvascular ECs, e.g. HDMVECs, HKMVECs and HRMVECs. This lower expression of sVEGFR-1 was also apparent on the mRNA level (B. Barleon, unpublished data). Up to now, we have no explanation for this lower expression level of sVEGFR-1 between macro- and microvascular-derived ECs. Although the isolation procedure for microvascular ECs is more difficult and the cells normally not as pure as HUVECs or HUAECs, we are not sure whether this is the reason. Because new blood vessels are mainly built up by microvascular ECs, expression of the endogenous VEGF-A antagonist sVEGFR-1 might be down-regulated to a certain extent in this cell type. It will be of interest to investigate this issue in more detail.

Although many growth factor receptors with tyrosine kinase activity exist as soluble forms, there is no clear concept regarding their physiological role [35, 36]. In the case of sVEGFR-1, it appears that this soluble receptor acts primarily as a specific endogenous antagonist of its membrane-bound counterparts, VEGFR-1 and VEGFR-2, by directly competing with the cell-surface receptors for ligand binding. This assumption is supported by the following findings: (i) rsVEGFR-1 is a specific inhibitor of VEGF-induced biological activities *in vitro* [15, 26]; (ii) a chimeric soluble VEGFR-1 fused to Fc suppresses retinal neovascularization *in vivo* [37]; (iii) a recombinant soluble form of the VEGFR-2 inhibits tumor growth as well as vascular density in a cutaneous tumor window chamber assay [38]; (iv) adenovirus-mediated *in vivo* regional delivery of a secreted form of the extracellular domain of VEGFR-1 can effectively inhibit regional tumor growth [39], (v) the HT-1080 human fibrosarcoma cell line expressing the native sVEGFR-1, inhibits tumor growth, metastasis and mortality rate in nude mice [40], and (vi) the A375v human melanoma cell line expressing the sVEGFR-1(5) inhibits tumor growth and tumor angiogenesis in nude mouse xenografts [41]. These results clearly demonstrate that sVEGFR-1 has the potential to inhibit VEGF action by a direct competition for ligand-binding *in vivo*.

However, the physiological role of sVEGFR-1 as well as the regulation of switching the transcription from the

transmembrane to the sVEGFR-1 is still not understood. Recently it was shown that in mouse placenta, the expression of both transmembrane and sVEGFR-1 changed during the progression of pregnancy [33]. They found by *in situ* hybridization that as gestation progressed there was a shift in the ratio from VEGFR-1 to sVEGFR-1. By growth-factor stimulated HUVECs we observed that both the full-length and the soluble form were up-regulated with an equal ratio on the mRNA as well as on the protein level. This opposite result might be explained by the different models used, *in vitro* versus *in vivo*. More interesting in this context is the finding that the two potent angiogenic factors, VEGF and bFGF, causing an enhanced secretion of sVEGFR-1 in cultured ECs, are both capable of up-regulating VEGFR-2 expression on the same cells ([42, 43]; B. Barleon, unpublished data). Furthermore, resting monocytes expressing low levels of VEGFR-1 mRNA and secreting only small amounts of sVEGFR-1, showed an increased secretion of sVEGFR-1 after activation with lipopolysaccharide. Mononuclear phagocytes, as represented by circulating monocytes and tissue macrophages, play important roles in host defense mechanisms and inflammatory disease processes [44]. The monocyte-derived macrophages appear to play a pivotal role in modulating this process. In addition, several lines of evidence also show that they are key regulators of angiogenesis [44, 45]. Thus, VEGF-A can contribute to angiogenesis of neoplastic and inflammatory tissues by acting directly on ECs as well as indirectly by favoring macrophage recruitment [12]. Our results indicate that both cell types, vascular ECs and monocytes/macrophages, might also be capable of regulating the VEGF-A-induced responses in both cell types by an increased secretion of sVEGFR-1. Very recently, sVEGFR-1 protein was found in CM of certain human hematopoietic cell lines [46]. Because VEGF has a number of effects on hematopoietic cells, e.g. regulation of hematopoietic cell proliferation [47], maturation of dendritic cells [48], and the protection of stem cells from apoptosis [49], the authors speculated that the sVEGFR-1 may play some roles in VEGF activity in normal and malignant hematopoietic cells. The mode of regulation might be the down-regulation of the VEGF-A-induced responses in both cell types by a negative feedback mechanism. Soluble VEGFR-1 may act in two different ways, first by regulation of the bioavailability of VEGF by sequestering the ligand, and second by formation of heterodimers with cell surface VEGFR-1 and -2 abolishing their signal transduction.

A role for sVEGFR-1 as a negative regulator of VEGF function is strongly supported by the recent finding of Hiratsuka et al. [50]. Whereas the VEGFR-1 null mutant mice embryos are lethal because of abnormal overgrowth of ECs [8], a VEGFR-1 lacking the tyrosine kinase domain is sufficient to allow embryonic development with normal angiogenesis and to overcome the lethal abnormality [50]. This result suggests a negative regulatory function of VEGFR-1 in VEGF-

induced angiogenesis. Since VEGFR-1 and sVEGFR-1 have a greater (more than 10-fold) affinity for VEGF than VEGFR-2, sVEGFR-1 can prevent activation of the membrane-bound VEGFR-1 and -2. Therefore, a reasonable explanation for this rescue of angiogenesis is that the extracellular domain of VEGFR-1, most likely the soluble one, is necessary and sufficient to absorb a significant amount of VEGF-A *in vivo*, resulting in a negative regulation of endothelial growth in embryonic angiogenesis. This result might also indicate that at least in the embryonic development the local secretion of sVEGFR-1 *in vivo* is sufficient to negatively interfere with the VEGF-A-induced biological activities.

In conclusion, we have shown that sVEGFR-1 is detectable in serum and plasma samples of normal healthy donors. Vascular ECs and monocytes most likely contribute to this sVEGFR-1 activity. The EC- and monocyte-derived sVEGFR-1 is capable of inhibiting the VEGF-A-induced responses in vascular ECs *in vitro*. However, additional investigations will be required to elucidate the exact physiological function(s) of sVEGFR-1 *in vivo* and to determine quantitatively whether the local amounts of sVEGFR-1 secreted by vascular ECs and/or monocytes/macrophages are sufficient to effectively interfere with the VEGF-A-induced biological activities *in vivo*. Furthermore, the screen of clinical relevant samples from patients with angiogenic diseases will be necessary to clearly show a physiological relevance of this sVEGFR-1 as a naturally occurring VEGF-A antagonist. In a collaboration with the group of Adrian Harris (Oxford, UK) we have used our sensitive ELISA to measure the amount of sVEGFR-1 in serum of patients with advanced renal cancer before and 1 month after anti-angiogenic therapy. Our results suggest that sVEGFR-1 may be of value in assessing anti-angiogenic treatments targeting different components of the vasculature or angiogenic therapy [51].

#### Acknowledgements

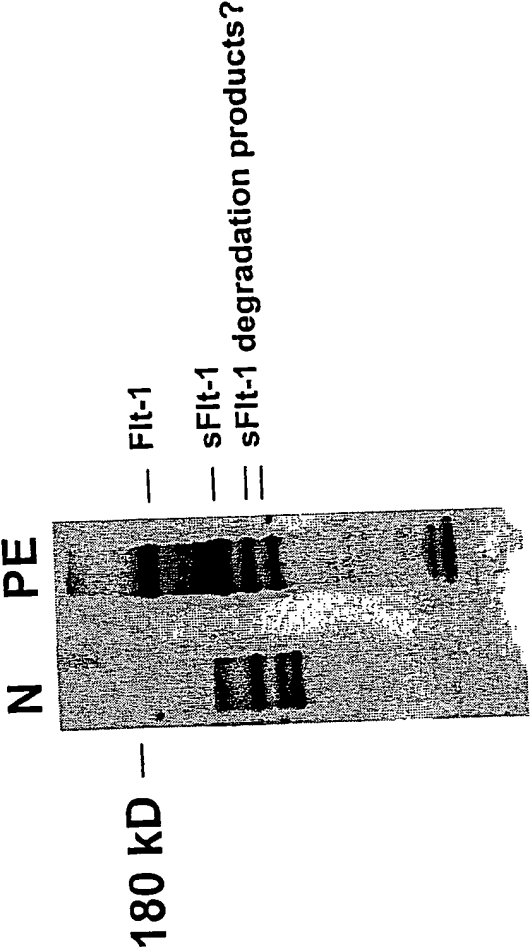
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Exhibit E



# Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia

See the related Commentary beginning on page 600.

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Preeclampsia, a syndrome affecting 5% of pregnancies, causes substantial maternal and fetal morbidity and mortality. The pathophysiology of preeclampsia remains largely unknown. It has been hypothesized that placental ischemia is an early event, leading to placental production of a soluble factor or factors that cause maternal endothelial dysfunction, resulting in the clinical findings of hypertension, proteinuria, and edema. Here, we confirm that placental soluble fms-like tyrosine kinase 1 (sFlt1), an antagonist of VEGF and placental growth factor (PlGF), is upregulated in preeclampsia, leading to increased systemic levels of sFlt1 that fall after delivery. We demonstrate that increased circulating sFlt1 in patients with preeclampsia is associated with decreased circulating levels of free VEGF and PlGF, resulting in endothelial dysfunction in vitro that can be rescued by exogenous VEGF and PlGF. Additionally, VEGF and PlGF cause microvascular relaxation of rat renal arterioles in vitro that is blocked by sFlt1. Finally, administration of sFlt1 to pregnant rats induces hypertension, proteinuria, and glomerular endotheliosis, the classic lesion of preeclampsia. These observations suggest that excess circulating sFlt1 contributes to the pathogenesis of preeclampsia.

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## Introduction

The circulating factor secreted by the placenta and the cause of the widespread endothelial dysfunction in preeclampsia has not yet been identified. Several candidates have been suggested, including homocysteine, TNF- $\alpha$ , soluble Fas ligand, anti-phospholipid antibodies, and oxidized lipid products, but none of these have been confirmed unequivocally in subsequent work (1, 2). Recently, neurokinin B was reported to be elevated in preeclampsia. Neurokinin B led to transient hypertension when administered intravenously to rats (3);

however, endothelial dysfunction and proteinuria were not reported. In an attempt to identify novel secreted factors playing a pathologic role in preeclampsia, we performed gene expression profiling of placental tissue from women with and without preeclampsia using Affymetrix U95A microarray chips and found soluble fms-like tyrosine kinase 1 (*sFlt1*) mRNA (GenBank accession number U01134) to be upregulated in preeclamptic placentas (data not shown). sFlt1, a splice variant of the VEGF receptor Flt1 lacking the transmembrane and cytoplasmic domains, acts as a potent VEGF and PlGF antagonist (4, 5). It is produced by a number of tissues, including the placenta (6, 7), but its physiologic role is unclear. Recently, both placental *sFlt1* expression (8) and sFlt1 levels in the amniotic fluid (9) have been noted to be elevated in preeclampsia; however, systemic levels of sFlt1 in preeclampsia have not yet been reported.

There is circumstantial evidence that antagonism of VEGF may have a role in hypertension and proteinuria. VEGF is a well-known promoter of angiogenesis; it also induces nitric oxide and vasodilatory prostacyclins in endothelial cells, suggesting a role in decreasing vascular tone and blood pressure (10, 11). VEGF has been

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**Conflict of interest:** Vikas P. Sukhatme is a consultant and equity holder in Ilex Oncology.

**Nonstandard abbreviations used:** soluble fms-like tyrosine kinase 1 (sFlt1); placental growth factor (PlGF); human umbilical vein endothelial cells (HUVEC); periodic acid Schiff (PAS); hemolysis, elevated liver function tests, and low platelets (HELLP).

implicated in glomerular healing, and anti-VEGF compounds have been found to increase apoptosis, impair glomerular capillary repair, and increase proteinuria in a rat model of mesangioproliferative nephritis (12). Furthermore, exogenous VEGF was found to accelerate renal recovery in rat models of glomerulonephritis and experimental thrombotic microangiopathy (13, 14). More recently, exogenous VEGF was shown to ameliorate post-cyclosporine-mediated hypertension, endothelial dysfunction, and nephropathy (15). Finally, in recent antiangiogenic clinical trials, VEGF signaling inhibitors have resulted in hypertension and proteinuria (16). Collectively, these data suggest that VEGF is important not only in blood pressure regulation but also in maintaining the integrity of the glomerular filtration barrier. We therefore hypothesized that excess circulating sFlt1 secreted by the placenta in preeclampsia leads to endothelial dysfunction, hypertension, and proteinuria by antagonizing circulating VEGF and PlGF. Here, we demonstrate that excess sFlt1 in patients with preeclampsia causes endothelial dysfunction and produces a syndrome of nephrotic-range proteinuria, hypertension, and glomerular endotheliosis when administered exogenously to animals. Although the primary trigger for abnormal placental development and excess sFlt1 production in preeclampsia remains speculative, our work suggests that excess sFlt1 alone may be sufficient to produce generalized endothelial dysfunction and some of the clinical phenotype noted in preeclampsia.

## Methods

**Reagents.** Human VEGF, rat VEGF, human PlGF, mouse PlGF, recombinant human sFlt1-Fc, and mouse sFlt1-Fc were purchased from R&D Systems (Minneapolis, Minnesota, USA). Human VEGF, human PlGF, human sFlt1, mouse sFlt1, and mouse sFlk1 ELISA kits were also purchased from R&D.

**Patients.** Preeclampsia was defined by (1) systolic blood pressure of more than 140 mmHg and diastolic blood pressure of more than 90 mmHg after 20 weeks' gestation in a previously normotensive patient (2), new-onset proteinuria (>300 mg of protein in a 24-hour urine collection or a random urine protein/creatinine ratio of >0.3), and (3) resolution of hypertension and proteinuria by 12 weeks postpartum. Patients with baseline hypertension, proteinuria, or renal disease were excluded. For the purposes of this study, patients were divided into groups with mild and severe preeclampsia on the basis of the recently published ACOG criteria (17). Healthy, normotensive pregnant women (the "normal" group) were included as controls; six patients with preterm deliveries (the "preterm" group) for other reasons were included as additional controls. Placental samples were obtained immediately after delivery. Four random samples were taken from each placenta, and RNA isolation was performed using Qiagen RNeasy Maxi Kit (Qiagen, Valencia, California, USA). Serum was collected from pregnant patients

at the time of delivery ( $t = 0$ ) and 48 hours after delivery ( $t = 48$ ) after obtaining informed consent. These experiments were approved by the institutional review board at the Beth Israel Deaconess Medical Center.

**Northern blots, ELISA, and Western blots.** Northern blot experiments were done as previously described (18). The *Flt1* probe used for Northern blots was a 500-bp fragment in the coding region from pUC 118 human *Flt1* cDNA (a gift from D Mukhopadhyay) and *GAPDH* cDNA was used as a normalization control. ELISA for human sFlt1, human VEGF, and human PlGF was performed according to the manufacturer's instructions (R&D Systems). Briefly, the various samples for ELISA measurement were diluted in 0.1% BSA/Tris-buffered saline and were incubated in a 96-well plate precoated with a capture antibody directed against VEGF, PlGF, or sFlt1 for 2 hours. The wells were then washed three times in 0.05% Tween 20/PBS and incubated with a secondary antibody against VEGF, PlGF, or sFlt1 conjugated to horseradish peroxidase for an additional 2 hours. The plates were then washed again three times, substrate solution containing  $H_2O_2$  and tetramethylbenzidine was added, and optical density was determined at 450 nm. All assays were done in duplicate, and the protein levels were calculated using a standard curve derived from known concentrations of the respective recombinant proteins. Western blots for PlGF expression in rat blood specimens were performed using a goat polyclonal antibody directed against mouse PlGF-2 (R&D Systems) using previously described methodology (18). Western blots and ELISA were used for verifying the expression of adenoviral-infected transgenes in the rat plasma as described elsewhere (19).

**Endothelial tube assay.** Growth factor-reduced Matrigel (7 mg/ml; Collaborative Biomedical Products, Bedford, Massachusetts, USA) was placed in the wells (100  $\mu$ l per well) of a prechilled 48-well cell-culture plate and incubated at 37°C for 30 minutes to allow polymerization. Human umbilical vein endothelial cells (HUVEC) (30,000 cells in 300  $\mu$ l of endothelial basal medium with no serum; Clonetics, Walkersville, Maryland, USA) were treated with 5% patient serum, plated onto the Matrigel-coated wells, and incubated at 37°C for 12–16 hours. Tube formation was then assessed through an inverted phase-contrast microscope at  $\times 4$  (Nikon Corporation, Tokyo, Japan), and tube length was quantitatively analyzed using the Simple PCI imaging analysis software (Compix Inc. Imaging Systems, Township Pennsylvania, USA).

**Renal microvascular reactivity experiments.** Microvascular reactivity experiments were done as described previously (20) using rat renal microvessels (internal diameter, 70–170  $\mu$ m). In all experimental groups, the relaxation responses of kidney microvessels were examined after precontraction of the microvessels with U46619 (thromboxane agonist) to 40–60% of their baseline diameter at a distending pressure of 40 mmHg. Once the steady-state tone was reached, the

responses to various reagents such as VEGF, PlGF, and sFlt1 were examined in a standardized order. All drugs were applied extraluminally.

**Animal model.** Both pregnant and nonpregnant Sprague-Dawley rats were injected with  $1 \times 10^9$  PFU of adenoviruses (Ad Fc, Ad sFlt1, or Ad sFlk1-Fc) by injection into the tail vein. These adenoviruses have been described elsewhere (19) and were generated at the Harvard Vector Core Laboratory. For the low-dose sFlt1 experiment,  $1 \times 10^8$  PFU of adenovirus expressing sFlt1 was used. Pregnant rats were injected on day 8 or 9 of pregnancy (early second trimester), and blood pressure was measured on day 16 or 17 of pregnancy (early third trimester). In nonpregnant animals, blood pressures were measured on day 8 after injection of the adenoviruses. Blood pressures were measured in the rats after anesthesia with sodium pentobarbital (60 mg/kg intraperitoneally). The carotid artery was isolated and cannulated with a 3-Fr high-fidelity microtip catheter connected to a pressure transducer (Millar Instruments, Houston, Texas, USA). Blood pressure was recorded and averaged over a 10-minute period. Blood, tissue, and urine samples were then obtained before euthanasia. We measured plasma levels on the day of blood pressure measurement (day 8 after injection of the adenoviruses), recognizing that 7–10 days after adenoviral injection corresponds to the peak level of expression of these proteins. Circulating sFlt1 and sFlk1 levels were confirmed initially by Western blotting (19) and then quantified using commercially available murine ELISA kits (R&D Systems). Urinary albumin was measured by standard dipstick and quantified by competitive enzyme-linked immunoassay as has been described elsewhere (21). Urinary creatinine was measured by a picric acid colorimetric procedure kit (Sigma-Aldrich, St. Louis, Missouri, USA).

**Statistical comparisons.** Results are presented as means  $\pm$  SEM, and comparisons between multiple groups were made using ANOVA. Significant differences are reported when  $P < 0.05$ .

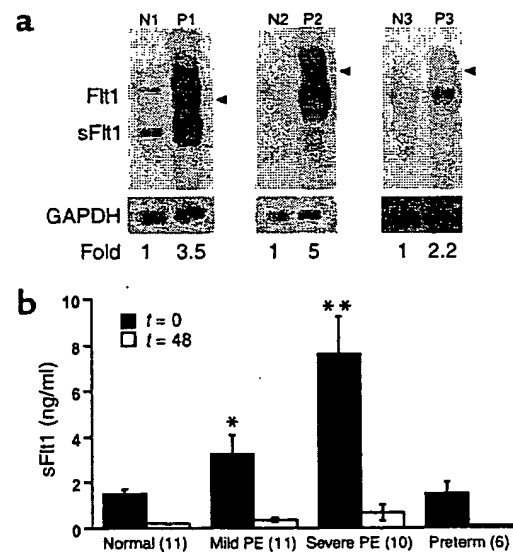
**Histology and electron microscopy.** Harvested kidneys from the rats were placed in Bouin's solution, paraffin embedded, sectioned, and stained with H&E, periodic acid Schiff (PAS), or Masson trichrome stain. For electron microscopy, renal tissue was fixed in glutaraldehyde and embedded in araldite-epon mixture; 1- $\mu$ m sections were cut, stained with methylene blue, and assessed before ultrastructural study. Immunofluorescence for fibrin deposits within the glomeruli was done using polyclonal anti-fibrin antibody (ICN Biochemicals, Aurora, Ohio, USA).

## Results

**Elevated placental sFlt1 production in preeclampsia.** We first confirmed increased placental sFlt1 mRNA by Northern analysis and found that both sFlt1 and Flt1 messages were upregulated in preeclamptic placentas (Figure 1a). We then measured maternal total sFlt1 serum levels by ELISA in 32 pregnant women with and with-

out preeclampsia (Figure 1b). The average serum level of sFlt1 was almost five times higher in patients with severe preeclampsia than in normotensive pregnant women. To exclude the possibility that this effect was due to the earlier gestational age of patients with preeclampsia, we also measured sFlt1 levels in gestational age-matched normotensive women delivering prematurely for other reasons and found no significant difference between this group and normotensive women with term pregnancies (Figure 1b).

**Decreased free VEGF and free PlGF in patients with preeclampsia.** sFlt1 is known to antagonize the proangiogenic molecules VEGF and PlGF by binding to them and preventing their interaction with their cell-surface receptors, Flt1 and KDR. We hypothesized that in preeclampsia, excess sFlt1 causes widespread endothelial dysfunction by interfering with the normal physiologic effects of VEGF and/or PlGF. If so, decreased levels of "free" or unbound VEGF and PlGF should correlate with clinical disease. We measured free VEGF and PlGF serum levels using commercially available ELISA kits that have previously been shown to measure



**Figure 1**

mRNA and protein expression of sFlt1 in preeclampsia. (a) mRNA expression of placental sFlt1 from three patients with preeclampsia (P1, P2, and P3) and three normotensive term pregnancies (N1, N2, and N3) were determined by Northern blot analysis. The higher band (7.5 kb) is the full-length Flt1 mRNA, and the lower, more abundant band (3.4 kb) is the alternatively spliced sFlt1 mRNA. GAPDH is included as a loading control, and the location of 28S is indicated by an arrowhead. Patients P1 and P2 had severe preeclampsia, whereas patient P3 had mild preeclampsia. (b) ELISA was performed for sFlt1 on serum from patients with mild preeclampsia (PE), severe preeclampsia and from normotensive pregnant women at term (normal) as described in Table 1. Patients with preterm deliveries were included as additional controls to rule out changes due to gestational age. The numbers of patients tested are shown in parentheses on the x-axis. Serum samples were collected before delivery ( $t = 0$ ) and 48 hours after delivery ( $t = 48$ ). \* $P < 0.05$  and \*\* $P < 0.01$  as compared with normotensive controls.

**Table 1**  
Clinical characteristics of the study patients

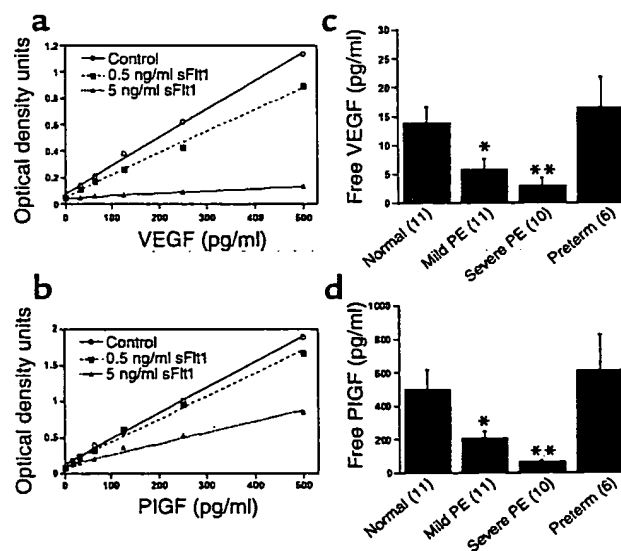
	Normal (n = 11)	Mild preeclampsia (n = 11)	Severe preeclampsia (n = 10)	Preterm (n = 6)
Maternal age (yrs)	34.5 ± 0.9	32.4 ± 1.6	30.2 ± 1.3	33.3 ± 1.5
Gestational age (wks)	38.8 ± 0.2	34.0 ± 1.0	31.2 ± 0.9	29.9 ± 1.7
Primiparous (%)	17%	73%	70%	80%
Systolic blood pressure (mmHg)	<140	151 ± 4.3	170 ± 5.6	<140
Diastolic blood pressure (mmHg)	<90	102 ± 2.6	103 ± 3.8	<90
Proteinuria (g protein/g creatinine)	<0.3	1.1 ± 0.2	7.0 ± 1.8	<0.3
Uric acid (mg/dl)	NA	6.4 ± 0.3	7.0 ± 0.2	NA
Hematocrit (%)	35.4 ± 0.6	34.6 ± 0.9	34.9 ± 1.5	34.4 ± 1.4
Platelet count	215 ± 18	214 ± 34	204 ± 27	220 ± 16
Creatinine (mg/dl)	0.6 ± 0.04	0.6 ± 0.03	0.5 ± 0.03	0.5

Values shown are means ± SEM. Of the six patients in the preterm group, four had preterm labor, one had intrauterine growth retardation, and one had placenta previa. NA, not available.

free VEGF and free PlGF (22, 23). We first confirmed that these ELISA kits measured only unbound VEGF or PlGF by performing a standard curve for VEGF and PlGF proteins in the presence of exogenous recombinant sFlt1. Figure 2 (a and b) shows that VEGF and PlGF levels were significantly decreased in the presence of recombinant sFlt1. Note that interference of sFlt1 with VEGF measurement was greater than that with PlGF measurement, because Flt1 binds to VEGF with a higher affinity (VEGF,  $K_d = 10\text{--}20\text{ pM}$ ; PlGF,  $K_d = 250\text{ pM}$ ) (24, 25). Using these ELISA systems, we then measured free VEGF and free PlGF in the serum of preeclamptic patients and in control women and confirmed that both free VEGF and free PlGF were significantly decreased in patients with preeclampsia (Figure 2, c and d). In fact, the decrease in levels of free VEGF and PlGF was proportionate to the rise in serum sFlt1 levels in these patients.

*Impaired angiogenesis due to excess sFlt1 in preeclamptic serum.* To address our hypothesis that excess circulating sFlt1 in patients with preeclampsia causes endothelial dysfunction and leads to an antiangiogenic state, we measured endothelial tube formation, an established in vitro model of angiogenesis. The conditions of the tube formation assay were adjusted so that normal HUVEC cells formed tubes only in the presence of exogenous growth factors such as VEGF or PlGF (data not shown). Under these conditions, we found that although serum from normotensive women induced endothelial cells to form regular tube-like structures, serum from those with preeclampsia inhibited tube formation (Figure 3). Notably, by 48 postpartum hours, this antiangiogenic effect had disappeared from the serum, suggesting that the inhibition of tubes noted with the serum from preeclamptic patients was due to a circulating factor released by the placenta. When sFlt1 was added to normotensive serum at concentrations noted in patients with preeclampsia, tube formation did not occur, mimicking the effects seen with serum from preeclamptic patients. Finally, when exogenous VEGF and PlGF were added to serum from preeclamptic patients, tube formation was restored (Figure 3). These results suggest that the antiangiogenic proper-

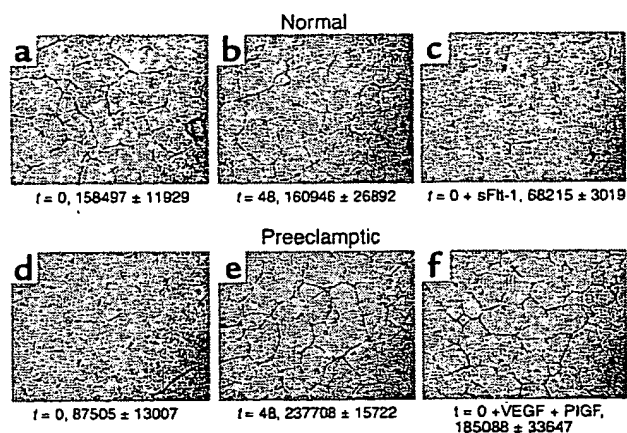
genic state, we measured endothelial tube formation, an established in vitro model of angiogenesis. The conditions of the tube formation assay were adjusted so that normal HUVEC cells formed tubes only in the presence of exogenous growth factors such as VEGF or PlGF (data not shown). Under these conditions, we found that although serum from normotensive women induced endothelial cells to form regular tube-like structures, serum from those with preeclampsia inhibited tube formation (Figure 3). Notably, by 48 postpartum hours, this antiangiogenic effect had disappeared from the serum, suggesting that the inhibition of tubes noted with the serum from preeclamptic patients was due to a circulating factor released by the placenta. When sFlt1 was added to normotensive serum at concentrations noted in patients with preeclampsia, tube formation did not occur, mimicking the effects seen with serum from preeclamptic patients. Finally, when exogenous VEGF and PlGF were added to serum from preeclamptic patients, tube formation was restored (Figure 3). These results suggest that the antiangiogenic proper-



**Figure 2**

Free VEGF and free PlGF levels are decreased in the serum of patients with preeclampsia. (a) Standard curve for recombinant human VEGF protein was generated in the absence (control) or in the presence of two different doses of recombinant human sFlt1-Fc using the ELISA kit for measurement of human VEGF protein, as described in Methods. (b) Standard curve for recombinant human PlGF protein was generated in the absence (control) or in the presence of two different doses of recombinant human sFlt1-Fc using the ELISA kit for measurement of human PlGF protein, as described in Methods. (c) Free VEGF levels (pg/ml) at the time of delivery ( $t = 0$ ) were determined by ELISA for the four patient groups described in Figure 1b and Table 1. (d) Free PlGF levels (pg/ml) at the time of delivery were determined by ELISA for the four patient groups described in Figure 1b and Table 1. The numbers of patients tested are shown in parentheses on the x-axis. \* $P < 0.05$  and \*\* $P < 0.01$  as compared with normotensive controls.





**Figure 3**  
Preeclampsia is an antiangiogenic state due to excess sFlt1. Endothelial tube assay was performed using serum from four normal pregnant controls and four patients with preeclampsia before and after delivery. A representative experiment from one normal control and one patient with preeclampsia is shown. (a) t = 0 (5% serum from a normal pregnant woman at term). (b) t = 48 (5% serum from a normal pregnant woman 48 hours after delivery). (c) t = 0 plus exogenous sFlt1 (10 ng/ml). (d) t = 0 (5% serum from a preeclamptic woman before delivery). (e) t = 48 (5% serum from a preeclamptic woman 48 hours after delivery). (f) t = 0 plus exogenous VEGF (10 ng/ml) and PlGF (10 ng/ml). The tube assay was quantified, and the mean tube length ± SEM in pixels is given at the bottom of each panel for all the patients analyzed. Recombinant human VEGF, human PlGF, and human sFlt1-Fc were used for the assays.

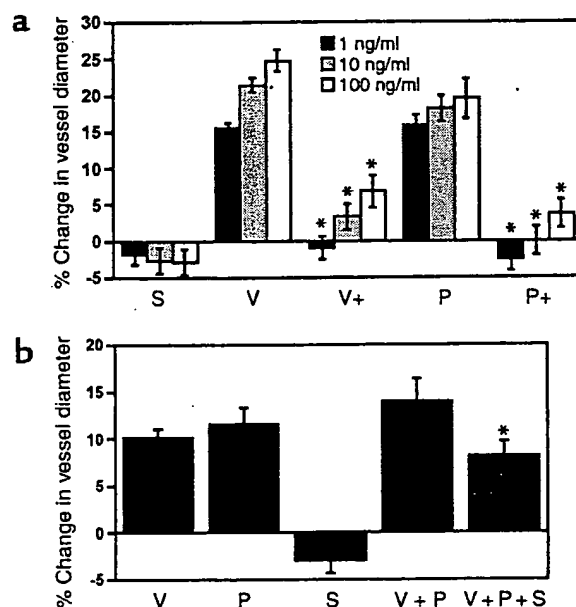
ties of serum from preeclamptic patients are due to blockade of VEGF and PlGF by endogenous sFlt1. Similar data were also obtained using primary human endothelial cells derived from the uterine microvasculature (data not shown).

**Inhibition of VEGF- and PlGF-induced vasodilation by sFlt1.** To assess the hemodynamic effects of circulating sFlt1, we performed a series of experiments using an in vitro assay for microvascular reactivity (20). We found that sFlt1 alone did not cause significant vasoconstriction. However, sFlt1 blocked the dose-dependent increase in vasodilation induced by VEGF or PlGF (Figure 4a). Data shown in Figure 4b confirmed that sFlt1 significantly inhibited VEGF- and PlGF-induced vasodilation at a level observed in patients with severe preeclampsia. These data suggest that circulating sFlt1 in preeclamptic patients might oppose physiologic vasorelaxation, thus contributing to hypertension.

**In vivo effects of sFlt1 on blood pressure and proteinuria.** On the basis of these results, we predicted that exogenous sFlt1 might produce hypertension and proteinuria in an animal model. Adenovirus expressing sFlt1 has been shown to produce sustained systemic sFlt1 levels associated with significant antitumor activity (19). This recombinant adenovirus encoding the murine sFlt1 gene product was injected into the tail vein of pregnant rats on day 8 or 9 of pregnancy (normal rat gestation, 21 days). Adenovirus encoding murine Fc protein in equivalent doses was used as a control to rule out non-

specific effects of adenoviruses. Flk1 (VEGF receptor-2) has been shown to bind VEGF but not PlGF (24). Hence, adenovirus encoding murine sFlt1-Fc transgene (soluble fusion protein of mouse VEGF receptor Flk1 ectodomain and mouse Fc protein) was chosen as an additional control to determine if the antagonism of VEGF alone would be sufficient to produce a phenotype. We measured intra-arterial blood pressure in the early third trimester (day 16 or 17), corresponding to the onset of hypertension in preeclampsia. These experiments were also completed in nonpregnant female rats to assess whether the effects observed with sFlt1 were dependent on the presence of the placenta.

Blood pressure and albuminuria in the different experimental groups are shown in Table 2. The mean sFlt1 level in rats injected with adenovirus expressing sFlt1 was  $215.5 \pm 81.2$  ng/ml, and the mean sFlt1 level was  $887.5 \pm 204.8$  ng/ml. Pregnant rats treated with sFlt1 had significant hypertension and heavy albuminuria, whereas both Fc and sFlt1-Fc pregnant control rats did not. Nonpregnant rats administered sFlt1 also developed hypertension and proteinuria. Notably, the sFlt1-Fc-treated nonpregnant rats developed hypertension and proteinuria, whereas the sFlt1-Fc-treated



**Figure 4**  
sFlt1 inhibits VEGF- and PlGF-induced vasodilation of renal microvessel. (a) The relaxation responses of rat renal arterioles to sFlt1, VEGF, and PlGF at three different doses were measured. S, sFlt1; V, VEGF; P, PlGF. V+ and P+ represent responses to VEGF and PlGF in the presence of sFlt1 at 100 ng/ml. A positive change reflects an increase in vessel diameter. (b) Microvascular relaxation responses were measured at physiological doses of VEGF (100 pg/ml), PlGF (500 pg/ml), sFlt1 (10 ng/ml), VEGF (100 pg/ml) plus PlGF (500 pg/ml), or VEGF (100 pg/ml) plus PlGF (500 pg/ml) plus sFlt1 (10 ng/ml). Each experiment was repeated in six different rat renal microvessels, and data are reported as means ± SEM. \*P < 0.05 as compared with VEGF plus PlGF. Reagents used for the assays were recombinant rat VEGF, mouse PlGF, and mouse sFlt1-Fc.

**Table 2**  
Blood pressure and proteinuria in rats

	<i>n</i>	Mean arterial pressure (mmHg)	Urine albumin/creatinine ratio (μg/mg)
Fc (pregnant)	5	75 ± 11	62 ± 21
sFlt1 (pregnant)	4	109 ± 19 <sup>A</sup>	6923 ± 658 <sup>B</sup>
sFlk1-Fc (pregnant)	4	73 ± 15	50 ± 32
Fc (nonpregnant)	5	89 ± 6	138 ± 78
sFlt1 (nonpregnant)	6	118 ± 13 <sup>A</sup>	12947 ± 2776 <sup>B</sup>
sFlk1-Fc (nonpregnant)	4	137 ± 2 <sup>A</sup>	2269 ± 669 <sup>B</sup>

Pregnant and nonpregnant rats were administered adenovirus expressing Fc (control), sFlt1, or sFlk1-Fc protein. Mean arterial blood pressure (diastolic plus one third of the pulse pressure in mmHg) ± SEM and mean urine albumin/creatinine ratio (micrograms of albumin per milligram of creatinine) ± SEM were measured 8 days after adenoviral administration corresponding to the early third trimester in the pregnant rats. <sup>A</sup>*P* < 0.05 and <sup>B</sup>*P* < 0.01 as compared with the control group (Fc). Mean plasma sFlt1 levels were 388 ng/ml (pregnant) and 101 ng/ml (nonpregnant) in the sFlt1-treated rats. Mean plasma sFlk1 levels were 775 ng/ml (pregnant) and 1000 ng/ml (nonpregnant) in the sFlk1-Fc-treated rats.

pregnant rats did not. In pregnancy, therefore, the antagonism of VEGF alone appears to be insufficient to produce preeclampsia, possibly owing to high levels of unopposed PlGF secreted by the placenta. In the nonpregnant state, in which PlGF is virtually absent (Figure 5), antagonism of VEGF alone is sufficient to disrupt the balance of pro- and antiangiogenic forces, so as to produce systemic effects.

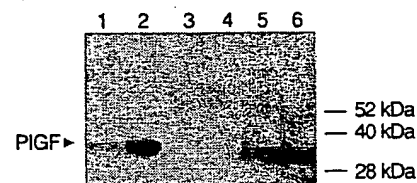
**Renal pathologic changes due to sFlt1.** Figure 6a shows the renal lesion that was observed in all sFlt1 treated pregnant rats: glomerular enlargement with occlusion of the capillary loops by swelling and hypertrophy of endocapillary cells (glomerular endotheliosis) (26). No segmental glomerulosclerosis or significant proliferation or vessel wall changes were observed. Electron microscopy of sFlt1 treated kidneys confirmed the glomerular changes seen on light microscopy (Figure 6b). Extensive capillary occlusion by intraluminal cells with swollen cytoplasm was seen. Podocytes showed protein resorption droplets with only focal foot-process effacement. Immunofluorescence studies of kidneys from sFlt1-treated rats showed focal deposition of fibrin within glomeruli (Figure 6b), changes that have been described as typical of the prepartum stage of human preeclampsia (27). The control Fc-treated rats (Figure 6, a and b) and sFlk1-Fc-treated pregnant rats did not show any renal pathology (Figure 6a). This constellation of renal pathologic findings noted in the sFlt1-treated animals is specific and represents the classic findings seen on renal biopsies in human preeclampsia (26, 28, 29). The sFlt1-treated nonpregnant and sFlk1-Fc-treated nonpregnant rats developed renal lesions similar to those in the sFlt1-treated pregnant rats, whereas the Fc-treated nonpregnant rats did not show any renal pathology (data not shown).

**Low-dose sFlt1 produces a milder phenotype.** Since the sFlt1 levels achieved in our initial experiments were significantly higher than levels observed in humans with preeclampsia (mean of 215.5 ng/ml in rats vs. 7.6 ng/ml in humans with severe disease), we conducted follow-

up experiments using lower doses of sFlt1. In these rats (*n* = 5), mean serum sFlt1 levels were comparable to those seen in preeclamptic women (7.3 ± 3.2 ng/ml). These animals also developed significant hypertension (mean arterial pressure, 119 ± 5 mmHg) and albuminuria (899 ± 286 μg of albumin per milligram of creatinine). Although the degree of albuminuria was much less than that observed in the high-dose experiments (Table 2), it was similar to what was noted in our patients with mild preeclampsia. The pathologic glomerular changes were also suggestive of a less severe phenotype, with endotheliosis and protein resorption droplets occurring in a focal and segmental pattern (Figure 6c).

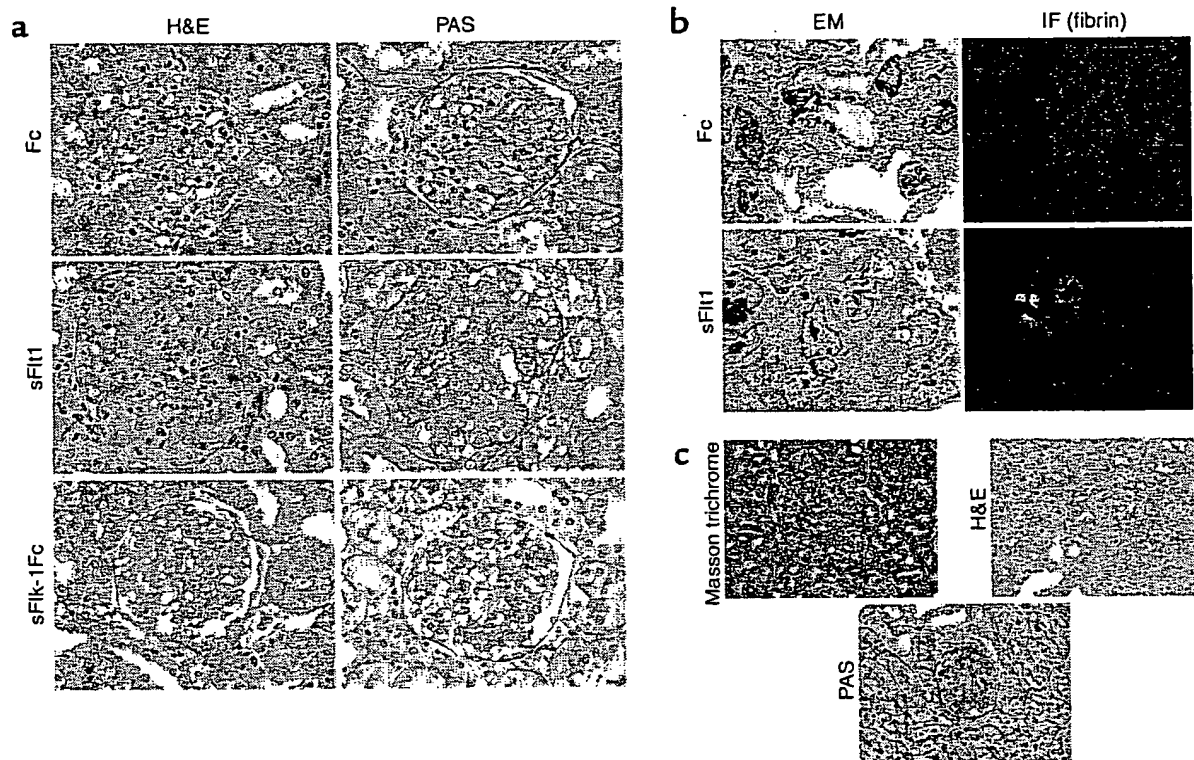
## Discussion

Several conclusions can be drawn from our findings. First, preeclampsia is associated with elevated circulating sFlt1 protein. It is likely that the excess sFlt1 production originates in the placenta, since we have shown that placental sFlt1 mRNA is upregulated in preeclampsia and that levels fall within 48 hours after delivery. Second, exogenously administered sFlt1 is sufficient to produce several of the clinical and pathological findings of preeclampsia in rats, including hypertension and glomerular endotheliosis. Third, the systemic effects of sFlt1 do not require the presence of pregnancy or the placenta, since hypertension and glomerular changes occurred in both nonpregnant and pregnant rats; this suggests a direct effect of sFlt1 on the maternal endothelium. Our work also suggests that sFlt1 acts through its antagonism of both VEGF and PlGF, since the VEGF antagonist sFlk1 did not produce the preeclampsia phenotype in pregnant rats. The observation that nonpregnant rats treated with sFlk1 did develop HTN and proteinuria is consistent with this hypoth-



**Figure 5**

Western blot analysis for PlGF expression in pregnant rats versus nonpregnant rats. Western blot analysis for PlGF levels in the systemic circulation of rats was performed using blood specimens from two nonpregnant and two pregnant rats (early third trimester) as described in Methods. Lanes 1 and 2 represent 1 ng and 10 ng of recombinant mouse PlGF protein used as a positive control. Twenty microliters of concentrated (10-fold) serum specimens from two nonpregnant rats (lanes 3 and 4) and two pregnant rats in the early third trimester (lanes 5 and 6) were used, and shown is a representative Western blot. The blot shows almost absent PlGF in the nonpregnant rats and expression of PlGF protein in pregnant rats.



**Figure 6** sFlt1 induces glomerular endotheliosis. (a) Histopathological analysis of renal tissue from one representative Fc-treated pregnant rat (upper panel), one sFlt1-treated pregnant rat (middle panel), and one sFlt1-Fc-treated pregnant rat (lower panel) is shown here. H&E stain shows capillary occlusion in the sFlt1 treated animal with enlarged glomeruli and swollen endothelial cells compared to Fc control animal and sFlt1-Fc control animals. PAS stain of the sFlt1-treated rat demonstrates PAS-negative swollen cytoplasm of endocapillary cells (endotheliosis). Numerous protein resorption droplets are also seen in the PAS section. These pathologic changes are absent in the Fc-treated rat as well as the sFlt1-Fc-treated pregnant rats. All light photomicrographs were taken at  $\times 60$  (original magnification). (b) Electron microscopy (EM) and immunofluorescence (IF) for fibrin was performed for the same rats shown in Figure 6a. Electron micrographs of glomeruli from an sFlt1-treated rat (lower panel) confirmed cytoplasmic swelling of the endocapillary cells. There is relative preservation of the podocyte foot processes and the basement membranes. Immunofluorescence for fibrin shows foci of fibrin deposition within the glomeruli of sFlt1-treated rats but not Fc-treated rats. The immunofluorescence pictures were taken at  $\times 40$  and the electron micrographs were taken at  $\times 2400$  (original magnification). All figures are reproduced at identical magnifications. (c) Histopathological analysis of one representative nonpregnant rat treated with low-dose sFlt1 is shown here. Low-power ( $\times 30$ , original magnification) Masson trichrome staining of renal tissue from the low-dose sFlt1-treated rat shows varying glomerular size representing focal endotheliosis. This degree of variation in glomerular involvement was only noted in the low-dose group. Higher-power H&E staining and PAS staining showed segmental endotheliosis and protein resorption droplets with preservation of basement membranes.

esis, since circulating PlGF is negligible in this setting. Finally, we have developed a novel experimental model resembling human preeclampsia, suitable for exploring both the pathophysiology of preeclampsia and for testing potential therapeutic compounds.

Emerging data on the role of VEGF in proteinuria lends validity to our findings. In a recent abstract describing new conditional knockout mice, reduction of VEGF production by podocytes alone led to massive proteinuria and glomerular endotheliosis (30). Additionally, VEGF-neutralizing antibodies in clinical cancer trials have resulted in proteinuria (16). These reports support the hypothesis that VEGF deficiency in the glomerulus, as may occur with excess sFlt1 in preeclampsia, produces proteinuria.

The role of VEGF in preeclampsia has received substantial attention. Several authors have reported

increased systemic VEGF levels in women with preeclampsia (31–34) while other authors have reported decreased levels (35–37), as we report here (Figure 2c). In reviewing the methodology of these studies carefully, we found that all studies reporting decreased VEGF (35–37) used a commercially available ELISA kit (R&D Systems), which, in fact, measures free (unbound) VEGF as previously shown by others (22, 23, 38). All studies reporting increased VEGF in preeclampsia used either radioimmunoassay or a non-R&D ELISA system, measuring total (bound and unbound) VEGF (31–34). Under many circumstances, these two entities would be interchangeable. However, in pregnancy, circulating sFlt1 is present at very high levels (the mean sFlt1 level in normal-term pregnancy was  $1.5 \pm 0.2$  ng/ml) (Figure 1b) as compared with the nonpregnant state, in

which sFlt1 levels are relatively low (the mean sFlt1 level in healthy female volunteers was  $0.15 \pm 0.04$  ng/ml) (39). Therefore, in normal pregnancy, and especially in preeclampsia where circulating levels of sFlt1 are extremely high, most VEGF is bound to circulating sFlt1 (40). Free VEGF levels, which more accurately reflect effective circulating VEGF, will thus be substantially lower than total VEGF levels. Similarly, the commercial PlGF ELISA kit from R&D Systems actually measures unbound (or free) PlGF (22), and other groups using this kit have demonstrated low circulating PlGF (37, 41), as we have confirmed here (Figure 2d). Seen in this light, the previously confusing and contradictory literature on VEGF supports our hypothesis that preeclampsia is characterized by normal to high total VEGF levels (perhaps induced by placental hypoxia) but low free VEGF and free PlGF levels, owing to a vast excess of sFlt1.

Several aspects of this work suggest that factors in addition to sFlt1 are likely to be playing a role in the pathogenesis of preeclampsia. Although serum sFlt1 levels were elevated in most patients with preeclampsia, a subset of patients in the mild preeclampsia group had only slightly elevated levels. Thus, sFlt1 may be causative in most but not all cases of preeclampsia in humans. Thrombocytopenia was not noted in our sFlt1-treated animals (data not shown), though it is invariably present in patients with hemolysis, elevated liver function tests, and low platelets (HELLP) syndrome, a variant of preeclampsia. This suggests that additional factors may be involved in HELLP syndrome. Work is in progress to identify such factors, which may be synergistic with sFlt1. It is interesting to note that the pathologic effects of sFlt1 were dose dependent; rats treated with low-dose sFlt1, with plasma levels similar to those seen in preeclamptic women, generally showed milder renal pathology as compared with rats treated with higher doses of sFlt1 (Figure 6c). A possible explanation is that sFlt1 may be one of several factors elaborated by the placenta that influence the severity of preeclampsia. It is also possible that recombinant adenoviral-linked sFlt1 has a less potent *in vivo* activity than endogenous sFlt1 present in human serum or that more prolonged, sustained levels of sFlt1 are required to produce severe disease.

Our work leaves many unanswered questions and paths for future work. Our data do not distinguish whether sFlt1 production by the placenta is a primary or secondary event. Hypoxia has been shown to increase sFlt1 production by placental cytotrophoblasts (22). If placental hypoxia is an early event in preeclampsia, sFlt1 release may occur as a secondary phenomenon. It has been proposed that placental angiogenesis is defective in preeclampsia, as evidenced by failure of the cytotrophoblasts to convert from an epithelial to an endothelial phenotype (referred to as pseudovasculogenesis) and invade maternal spiral arteries (42). It seems plausible that angiogenic molecules such as VEGF, PlGF, and sFlt1 may be impor-

tant regulators of early placental development and pseudovasculogenesis. In fact, it has recently been shown that exogenous sFlt1 inhibits placental cytotrophoblast invasion *in vitro* (8). Thus, excess placental sFlt1, in addition to its direct effect on the maternal endothelium in the third trimester, may also play a more primary role in deranged placental development in preeclampsia. In our sFlt1-treated rats, we did not observe the placental pathologic changes typical of preeclampsia, such as placental infarcts and shallow spiral-artery invasion (data not shown). However, this may reflect the fact that sFlt1 protein was administered in the early second trimester, after spiral-artery invasion had already been established. Future studies in which exogenous sFlt1 is given earlier in pregnancy (i.e., from the first trimester) should clarify this issue, and the role of sFlt1 in placental cytotrophoblast differentiation and development should continue to be explored.

Little is known about the regulation of transcription and splicing of *Flt1/sFlt1*. Alternative splicing has been identified as a key regulatory step in *sFlt1* production (6). By Northern analysis, however, it appears that both *Flt1* and *sFlt1* are proportionally increased in preeclamptic placenta (Figure 1a), suggesting that upregulation of *sFlt1* is not occurring at the level of alternative splicing but at the level of transcription or mRNA stability. Future work investigating the regulation of *sFlt1* production may clarify these mechanisms.

Our study has other limitations. Although the rapid decline in sFlt1 levels after delivery and the upregulation of placental sFlt1 mRNA strongly suggest a placental origin of the sFlt1, future studies (for example, measuring sFlt1 levels in the umbilical vein and artery) are needed to show this conclusively. Tissue levels and activity of VEGF/PlGF signaling are not addressed by this study or other current literature. Studies looking at tissue VEGF or PlGF signaling in preeclampsia should clarify the role of sFlt1 in the pathogenesis even further. In our measurement of blood pressure, we used anesthetized animals; this is known to have blood pressure-lowering effects. Although sFlt1-treated animals had significantly higher mean arterial pressures as compared with controls, it is possible that noninvasive blood pressure monitoring of these animals might produce a even more dramatic change in blood pressure.

Our findings have important implications both for the diagnosis and therapy of preeclampsia and for the use of VEGF/PlGF signaling inhibitors in other diseases, such as cancer. If sFlt1 overexpression occurs early in pregnancy, it might serve as a diagnostic marker in patients at high risk for the development of preeclampsia. Currently, there is no specific treatment for preeclampsia, and severe cases often require premature delivery of the infant. If excess sFlt1 plays a causative role in preeclampsia, antagonizing its effects may ameliorate symptoms. For example, exogenous VEGF and/or PlGF therapy might reverse the endothe-

lial dysfunction noted in these patients, as suggested in our in vitro angiogenesis assays (Figure 3). Therapeutic strategies using small-molecule compounds aimed at shifting the angiogenesis balance in favor of proangiogenic molecules might allow delivery to be safely postponed. For example, nicotine has been shown to have proangiogenic properties by inducing endogenous VEGF (43). Moreover, it is well-known that cigarette smoking is associated with a lower incidence of preeclampsia (44, 45). Smoking has also been shown to lower sFlt1 levels in humans (46). Thus, short term use of nicotine in cases of severe preeclampsia might be an effective treatment. This work also has implications for anti-VEGF or anti-PlGF compounds currently undergoing clinical trials for the treatment of cancer and other disorders (19, 47). The finding of proteinuria and hypertension in nonpregnant rats treated with antagonists to VEGF and PlGF raises concerns over the safety of these agents in humans, and specific monitoring for renal and vascular side effects should be considered. On the other hand, the occurrence of hypertension and/or proteinuria may signal a shift in systemic angiogenic activity and might correlate with response to therapy.

In summary, our findings suggest that excess placental production of sFlt1 contributes to the hypertension, proteinuria, and glomerular endotheliosis noted in patients with preeclampsia. Antagonizing or overwhelming endogenous sFlt1 may be a promising therapeutic approach for these patients. A clearer understanding of sFlt1 gene regulation and splicing and its role in placental and systemic vascular function may lead to better insights into the pathogenesis, treatment, and prevention of preeclampsia.

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